



MONOCLONAL AND CHIMERIC ANTIBODIES SPECIFIC FOR
HUMAN TUMOR NECROSIS FACTOR

08 013413

BACKGROUND OF THE INVENTION

This application is a continuation of each of U.S. application serial No. 07/943,852, filed September 11, 1992, U.S. application serial No. 07/853,606, filed March 18, 1992, and U.S. application serial No. 07/670,827, filed March, 18, 1991. U.S. application serial No. 07/943,852, filed September 11, 1992, is a continuation of U.S. application serial No. 07/853,606, filed March 18, 1992, which is a continuation of U.S. application serial No. 07/670,827, filed March, 18, 1991, now abandoned, all of which applications are entirely incorporated herein by reference.

FIELD OF THE INVENTION

The present invention in the field of immunology and medicine relates to antibodies and nucleic acid encoding therefor, which antibodies are specific for human tumor necrosis factor-alpha (hTNF α) and to pharmaceutical and diagnostic compositions and production, diagnostic and therapeutic methods thereof.

DESCRIPTION OF THE BACKGROUND ART

Tumor Necrosis Factor: The cytokine known as tumor necrosis factor- α (TNF α ; also termed cachectin) is a protein secreted primarily by monocytes and macrophages in response to endotoxin or other stimuli as a soluble homotrimer of 17 kD protein subunits (Smith, R.A. *et al.*, J. Biol. Chem. 262:6951-6954 (1987)). A membrane-bound 26 kD precursor form of TNF has also been described (Kriegler, M. *et al.*, Cell 53:45-53 (1988)). For reviews of TNF, see Beutler, B. *et al.*, Nature 320:584 (1986), Old, L.J., Science 230:630 (1986), and Le, J. *et al.*, Lab. Invest. 56:234 (1987). TNF was originally discovered in the serum of animals injected sequentially with a bacterial vaccine (bacillus Calmette-Guerin, BCG) and endotoxin (Carswell, E.A. *et al.*, Proc. Natl. Acad. Sci. USA 72:3666 (1975)).

The expression of the gene encoding TNF α is not limited to cells of the monocyte/macrophage family. Several human non-monocytic tumor cell lines were shown to produce TNF

(Rubin, B.Y. et al., J. Exp. Med. 164:1350 (1986); Spriggs, D. et al., Proc. Natl. Acad. Sci. USA 84:6563 (1987)). TNF is also produced by CD4⁺ and CD8⁺ peripheral blood T lymphocytes, and by various cultured T and B cell lines (Cuturi, M.C., et al., J. Exp. Med. 165:1581 (1987); Sung, S.-S.J. et al., J. Exp. Med. 168:1539 (1988)).

Accumulating evidence indicates that TNF is a regulatory cytokine with pleiotropic biological activities. These activities include: inhibition of lipoprotein lipase synthesis ("cachectin" activity) (Beutler, B. et al., Nature 316:552 (1985)), activation of polymorphonuclear leukocytes (Klebanoff, S.J. et al., J. Immunol. 136:4220 (1986); Perussia, B., et al., J. Immunol. 138:765 (1987)), inhibition of cell growth or stimulation of cell growth (Vilcek, J. et al., J. Exp. Med. 163:632 (1986); Sugarman, B.J. et al., Science 230:943 (1985); Lachman, L.B. et al., J. Immunol. 138:2913 (1987)), cytotoxic action on certain transformed cell types (Lachman, L.B. et al., supra; Darzynkiewicz, Z. et al., Canc. Res. 44:83 (1984)), antiviral activity (Kohase, M. et al., Cell 45:659 (1986); Wong, G.H.W. et al., Nature 323:819 (1986)), stimulation of bone resorption (Bertolini, D.R. et al., Nature 319:516 (1986); Saklatvala, J., Nature 322:547 (1986)), stimulation of collagenase and prostaglandin E2 production (Dayer, J.-M. et al., J. Exp. Med. 162:2163 (1985)); and immunoregulatory actions, including activation of T cells (Yokota, S. et al., J. Immunol. 140:531 (1988)), B cells (Kehrl, J.H. et al., J. Exp. Med. 166:786 (1987)), monocytes (Philip, R. et al., Nature 323:86 (1986)), thymocytes (Ranges, G.E. et al., J. Exp. Med. 167:1472 (1988)), and stimulation of the cell-surface expression of major histocompatibility complex (MHC) class I and class II molecules (Collins, T. et al., Proc. Natl. Acad. Sci. USA 83:446 (1986); Pujol-Borrell, R. et al., Nature 326:304 (1987)).

TNF is noted for its pro-inflammatory actions which result in tissue injury, such as induction of procoagulant activity on vascular endothelial cells (Poher, J.S. et al., J. Immunol. 136:1680 (1986)), increased adherence of neutrophils and lymphocytes (Poher, J.S. et al., J. Immunol. 138:3319 (1987)), and stimulation of the release of platelet activating

factor from macrophages, neutrophils and vascular endothelial cells (Camussi, G. et al., J. Exp. Med. 166:1390 (1987)).

Recent evidence implicates TNF in the pathogenesis of many infections (Cerami, A. et al., Immunol. Today 9:28 (1988)),
5 immune disorders, neoplastic pathology, e.g., in cachexia accompanying some malignancies (Oliff, A. et al., Cell 50:555 (1987)), and in autoimmune pathologies and graft-versus host pathology (Piguet, P.-F. et al., J. Exp. Med. 166:1280 (1987)).
The association of TNF with cancer and infectious pathologies is
10 often related to the host's catabolic state. A major problem in cancer patients is weight loss, usually associated with anorexia. The extensive wasting which results is known as "cachexia" (Kern, K.A. et al. (J. Parent. Enter. Nutr. 12:286-298 (1988))). Cachexia includes progressive weight loss,
15 anorexia, and persistent erosion of body mass in response to a malignant growth. The fundamental physiological derangement may be related to a decline in food intake relative to energy expenditure. The cachectic state is thus associated with significant morbidity and is responsible for the majority of
20 cancer mortality. A number of studies have suggested that TNF is an important mediator of the cachexia in cancer, infectious pathology, and in other catabolic states.

TNF is thought to play a central role in the pathophysiological consequences of Gram-negative sepsis and
25 endotoxic shock (Michie, H.R. et al., Br. J. Surg. 76:670-671 (1989); Debets, J.M.H. et al., Second Vienna Shock Forum, p.463-466 (1989); Simpson, S.Q. et al., Crit. Care Clin. 5:27-47 (1989)), including fever, malaise, anorexia, and cachexia. Endotoxin is a potent monocyte/macrophage activator which
30 stimulates production and secretion of TNF (Kornbluth, S.K. et al., J. Immunol. 137:2585-2591 (1986)) and other cytokines. Because TNF could mimic many biological effects of endotoxin, it was concluded to be a central mediator responsible for the clinical manifestations of endotoxin-related illness. TNF and
35 other monocyte-derived cytokines mediate the metabolic and neurohormonal responses to endotoxin (Michie, H.R. et al., N. Eng. J. Med. 318:1481-1486 (1988)). Endotoxin administration to human volunteers produces acute illness with flu-like symptoms including fever, tachycardia, increased metabolic rate and

stress hormone release (Revhaug, A. et al., Arch. Surg. 123:162-170 (1988)). Elevated levels of circulating TNF have also been found in patients suffering from Gram-negative sepsis (Waage, A. et al., Lancet 1:355-357 (1987); Hammerle, A.F. et al., Second Vienna Shock Forum p. 715-718 (1989); Debets, J.M.H. et al., Crit. Care Med. 17:489-497 (1989); Calandra, T. et al., J. Infec. Dis. 161:982-987 (1990)). Treatment of cancer patients with TNF (because of its tumoricidal action) revealed that doses greater than 545 $\mu\text{g}/\text{m}^2/24\text{hr}$ caused alterations similar to those induced by injection of endotoxin (4 ng/kg) into healthy humans (Michie, H.R. et al., Surgery 104:280-286 (1988)), supporting TNF's role as the principal host mediator of septic and endotoxemic responses. Chronic intravenous TNF infusion into humans or rats was associated with anorexia, fluid retention, acute phase responses, and negative nitrogen balance (i.e., classic catabolic effects), leading to the conclusion that TNF may be responsible for many of the changes noted during critical illness (Michie, H.R. et al., Ann. Surg. 209:19-24 (1989)).

Passive immunotherapy directed at neutralizing TNF may have a beneficial effect in Gram-negative sepsis and endotoxemia, based on the increased TNF production and elevated TNF levels in these pathology states, as discussed above.

Antibodies to a "modulator" material which was characterized as cachectin (later found to be identical to TNF) were disclosed by Cerami et al. (EPO Patent Publication 0212489, March 4, 1987). Such antibodies were said to be useful in diagnostic immunoassays and in therapy of shock in bacterial infections. Rubin et al. (EPO Patent Publication 0218868, April 22, 1987) disclosed monoclonal antibodies to human TNF, the hybridomas secreting such antibodies, methods of producing such antibodies, and the use of such antibodies in immunoassay of TNF. Yone et al. (EPO Patent Publication 0288088, October 26, 1988) disclosed anti-TNF antibodies, including mAbs, and their utility in immunoassay diagnosis of pathologies, in particular Kawasaki's pathology and bacterial infection. The body fluids of patients with Kawasaki's pathology (infantile acute febrile mucocutaneous lymph node syndrome; Kawasaki, T., Allergy 16:178 (1967); Kawasaki, T., Shonica (Pediatrics) 26:935 (1985)) were

said to contain elevated TNF levels which were related to progress of the pathology (Yone et al., supra).

Other investigators have described mAbs specific for recombinant human TNF which had neutralizing activity in vitro (Liang, C-M. et al. (Biochem. Biophys. Res. Comm. 137:847-854 (1986); Meager, A. et al., Hybridoma 6:305-311 (1987); Fendly et al., Hybridoma 6:359-369 (1987); Bringman, T.S. et al., Hybridoma 6:489-507 (1987); Hirai, M. et al., J. Immunol. Meth. 96:57-62 (1987); Moller, A. et al. (Cytokine 2:162-169 (1990)).

Some of these mAbs were used to map epitopes of human TNF and develop enzyme immunoassays (Fendly et al., supra; Hirai et al., supra; Moller et al., supra) and to assist in the purification of recombinant TNF (Bringman et al., supra). However, these studies do not provide a basis for producing TNF neutralizing antibodies that can be used for in vivo diagnostic or therapeutic uses in humans, due to immunogenicity, lack of specificity and/or pharmaceutical suitability.

Neutralizing antisera or mAbs to TNF have been shown in mammals other than man to abrogate adverse physiological changes and prevent death after lethal challenge in experimental endotoxemia and bacteremia. This effect has been demonstrated, e.g., in rodent lethality assays and in primate pathology model systems (Mathison, J.C. et al., J. Clin. Invest. 81:1925-1937 (1988); Beutler, B. et al., Science 229:869-871 (1985); Tracey, K.J. et al., Nature 330:662-664 (1987); Shimamoto, Y. et al., Immunol. Lett. 17:311-318 (1988); Silva, A.T. et al., J. Infect. Dis. 162:421-427 (1990); Opal, S.M. et al., J. Infect. Dis. 161:1148-1152 (1990); Hinshaw, L.B. et al., Circ. Shock 30:279-292 (1990)).

Putative receptor binding loci of hTNF has been disclosed by Eck and Sprang (J. Biol. Chem. 264(29), 17595-17605 (1989), who identified the receptor binding loci of TNF- α as consisting of amino acids 11-13, 37-42, 49-57 and 155-157. PCT application WO91/02078 (priority date of August 7, 1989) discloses TNF ligands which can bind to monoclonal antibodies having the following epitopes: at least one of 1-20, 56-77, and 108-127; at least two of 1-20, 56-77, 108-127 and 138-149; all of 1-18, 58-65, 115-125 and 138-149; all of 1-18, and 108-128; all of 56-79, 110-127 and 135- or 136-155; all of 1-30, 117-128

and 141-153; all of 1-26, 117-128 and 141-153; all of 22-40, 49-96 or -97, 110-127 and 136-153; all of 12-22, 36-45, 96-105 and 132-157; all of both of 1-20 and 76-90; all of 22-40, 69-97, 105-128 and 135-155; all of 22-31 and 146-157; all of 22-40 and 49-98; at least one of 22-40, 49-98 and 69-97, both of 22-40 and 70-87.

To date, experience with anti-TNF mAb therapy in humans has been limited. In a phase I study, fourteen patients with severe septic shock were administered a neutralizing mouse anti-TNF mAb in a single dose from 0.4-10 mg/kg (Exley, A.R. et al., Lancet 335:1275-1277 (1990)). However, seven of the fourteen patients developed a human anti-murine antibody response to the treatment, which treatment suffers from the known problems due to immunogenicity from the murine heavy and light chain portions of the antibody. Such immunogenicity causes decrease effectiveness of continued administration and can render treatment ineffective due to the increased immune rejection response in patients undergoing diagnostic or therapeutic administration of mouse anti-TNF antibodies.

Administration of murine TNF mAb to patients suffering from severe graft vs. host pathology has also been reported (Herve, P. et al., Lymphoma Res. 9:591 (1990)).

Accordingly, there is a need to provide novel TNF antibodies that overcome the problems of murine antibody immunogenicity and which provide reduced immunogenicity and increased neutralization activity.

SUMMARY OF THE INVENTION

It is an object of the present invention to overcome the deficiencies of the background art.

It is also an object of the present invention to provide anti-tissue necrosis factor (TNF) murine antibodies and chimeric antibodies, and fragments and regions thereof, which inhibit or neutralize TNF biological activity *in vivo* and are specific for human tumor necrosis factor-alpha (hTNF α). In a preferred embodiment, anti-TNF antibodies of the present invention competitively inhibit the binding of A2 antibodies to TNF.

In another preferred embodiment, anti-TNF antibodies of the present invention bind an epitope of at least 5 amino acids of residues 87-108 or both of residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1), but which do not bind known or putative
5 receptor binding portions of TNF, such as amino acid sequences 1-20, 11-13, 37-42, 49-57 or 155-157 of TNF (of SEQ ID NO:1).

Such anti-TNF antibodies, fragments and regions thereof of the present invention include those produced both from hybridomas, such as murine-murine hybridomas, and from
10 recombinant cells expressing heterologous nucleic acid encoding at least variable regions, such as murine-human chimeric antibodies, of such TNF specific antibodies, fragments or regions of the present invention.

Is a further object of the present invention to
15 provide anti-TNF antibodies, fragments, and regions using hybridoma and genetic engineering technologies to provide useful products for the *in vivo* treatment and diagnosis of human pathologies associated with TNF, as described above.

It is a further object of the present invention to
20 provide antigenic polypeptides of hTNF α which correspond to portions of TNF- α that, when epitopes of these polypeptides are bound by TNF-specific antibodies, fragments or regions which neutralize or inhibit the biological activity of TNF- α *in vivo*. It is another object of the present invention to provide
25 antibodies generated from hybridomas or recombinant hosts that bind specific epitopes of polypeptides corresponding to at least 5 amino acids selected from residues 87-108 or both residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1), as shown in Figure 15.

It is another object of the present invention to
30 provide anti-TNF α antibodies, fragments and regions which have TNF α inhibiting and/or neutralizing activity *in vivo*, and can be provided as pharmaceutical and diagnostic compositions for the diagnosis and treatment of TNF α -related pathologies, including
35 cachexia, acute and chronic infectious and parasitic processes, such as bacterial, viral and fungal infections, acute and chronic inflammatory and immune processes, including autoimmune pathology, alcohol-induced hepatitis, neoplastic pathology and the like. Preferred for therapeutic use are high affinity

anti-TNF α antibodies, including recombinantly and hybridoma produced monoclonal and chimeric antibodies, according to the present invention, which inhibit or neutralize human TNF- α with an *in vivo* inhibitory dose-50 (ID50) of at least about 1 μ g/ml, more preferably at least about 100 ng/ml, most preferably at least about 15 ng/ml.

According to another aspect of the present invention, anti-TNF α mAbs or chimeric mAbs, or TNF α -binding fragments thereof, which are particularly useful in diagnostic methods for detecting human TNF α in patients suspected of suffering from conditions associated with TNF α production, including methods wherein high affinity murine and/or chimeric anti-TNF α antibodies of the present invention are contacted with biological materials from a patient and an antigen-antibody reaction detected. Also included are kits for detecting TNF α in biological fluids comprising high affinity murine and/or chimeric anti-TNF α antibodies or fragments of the present invention, preferably in detectably labeled form.

The chimeric antibodies of the present invention embody a combination of the advantageous characteristics of mAbs. Like mouse mAbs, they can recognize and bind to human TNF; however, unlike mouse mAbs, the "human-specific" properties of the chimeric antibodies lower the likelihood of an immune response to the antibodies, and result in prolonged survival in the circulation through reduced clearance. Moreover, using the methods disclosed in the present invention, the constant region of any desired human immunoglobulin isotype can be combined with the desired antigen combining site.

In one embodiment, the present invention is directed to a high affinity mouse mAb, designated A2, which is specific for human TNF α . The antibody may be in detectably labeled form. In another embodiment, polypeptide portions of hTNF α are provided that, when bound as part of an intact TNF α molecule by antibodies or fragments specific for epitopes included in these peptide portions, inhibit or neutralize TNF α activity *in vivo*.

It is also an object of the present invention to provide chimeric immunoglobulin chains, either heavy (H) or light (L), having variable or constant regions, with specificity toward one or more epitopes of TNF, preferably an epitope of at

least 5 amino acids of residues 87-107, or a combination of both of 59-80 and 87-108, of hTNF α (of SEQ ID NO:1). In a preferred embodiment, the epitopic amino acids do not include amino acids from residues from at least one of 11-13, 37-42, 49-57 or 155-157 of hTNF α (of SEQ ID NO:1). A human/murine chimeric immunoglobulin chain contains a constant (C) region substantially similar to that present in a natural human immunoglobulin, and a variable (V) region, preferably non-human, having high affinity and the desired specificity for a TNF epitope. The invention also provides antibodies, fragments, and regions having chimeric H and L chains associated so that the overall molecule exhibits the desired antigen recognition and binding properties.

Specifically, the present invention is also directed to a chimeric antibody comprising two light chains and two heavy chains, each of the chains comprising at least part of a human constant region and at least part of a variable (V) region of non-human origin having specificity to human TNF α , said antibody binding with high affinity to a inhibiting and/or neutralizing epitope of human TNF α . The invention is also includes a fragment or a derivative such an antibody. Preferably, the V region is of non-human origin, most preferably of murine origin. In a preferred embodiment, the V region is derived from, or binds epitopes of the A2 mAb. In another preferred embodiment, a chimeric antibody is provided which binds epitopes of the antibody designated chimeric A2 (cA2), or a chimeric human-mouse anti-TNF mAb that competitively inhibits the binding of cA2 to TNF α .

Preferably, the chimeric antibody inhibits or neutralizes human TNF α *in vivo* with an ID50 of at least about 1 μ g/ml, more preferably at least about 100 ng/ml, most preferably at least about 15, 30, 50, or 80 ng/ml.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing dose dependent binding of mouse mAb A2 to human TNF α .

Figure 2 is a graph showing lack of recognition of heat-inactivated human TNF α by mAb A2.

Figure 3 is a graph showing neutralization of in vitro TNF cytotoxicity by murine A2. Control: murine IgG1 anti-lipid A mAb (8A1) with natural human TNF. Average absorbance values for controls were as follows: no TNF added = 1.08; natural TNF, no antibody = 0.290; and recombinant TNF, no antibody = 0.500.

Figure 4 is a graph showing that mAb A2 and chimeric A2 do not inhibit or neutralize human lymphotoxin (TNF β).

Figure 5 is a graph showing that mAbs murine A2 and chimeric CA2 do not inhibit or neutralize murine TNF α .

Figures 6 and Figure 7 are graphs showing that mAb A2 inhibits or neutralizes TNF produced by chimpanzee monocytes and rhTNF α .

Figure 8 provides schematic diagrams of the plasmids used for expression of the chimeric H (pA2HG1apgpt) and L (pA2HuKapgpt) chains of the chimeric A2 antibody.

Figure 9 is a graph showing results of a cross-blocking epitope ELISA with murine A2 (mA2) and chimeric (cA2) antibody competitors.

Figure 10 is a graph of a Scatchard analysis of ¹²⁵I-labelled mAb A2 (mA2) and chimeric A2 (cA2) binding to recombinant human TNF α immobilized on a microtiter plate. Each K_a value was calculated from the average of two independent determinations.

Figure 11 is a graph showing neutralization of TNF cytotoxicity by chimeric A2. The control is a chimeric mouse/human IgG1 anti-platelet mAb (7E3) reacting with natural human TNF. Average absorbance values for controls were: no TNF added = 1.08; natural TNF, no Ab = 0.290; and recombinant TNF, no Ab = 0.500.

Figure 12 is a graph showing in vitro neutralization of TNF-induced ELAM-1 expression by chimeric A2. The control is a chimeric mouse/human IgG1 anti-CD4 antibody.

Figure 13 is an amino acid sequence of human TNF as SEQ ID NO:1.

Figure 14A is a graphical representation of epitope mapping of chimeric mAb cA2 indicating relative binding of cA2 to human TNF peptide pins.

Figure 14B is a graphical representation of epitope mapping of chimeric mAb cA2 indicating relative binding of cA2 to human TNF peptide pins in the presence of human TNF.

Figure 15 is an amino acid sequence of human TNF showing sequences having portions of epitopes recognized by cA2.

Figure 16A is a representation of a space filling model of a human TNF monomer.

Figure 16B is a representation of a space filling model of two noncontiguous peptide sequences of human TNF recognized by cA2.

Figure 17A is a nucleic acid sequence of a cloned cA2 variable region (SEQ ID NO:2).

Figure 17B is a nucleic acid sequence of a cloned cA2 constant region (SEQ ID NO:3).

Figure 18 is a graphical representation of the early morning stiffness for the five patients in group I, and the four patients in group II is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent decrease or greater in early morning stiffness, which persisted for greater than 40 days.

Figure 19 is a graphical representation of the assessment of pain using a visual analogue scale for the five patients in group I, and the four patients in group II, is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 60 to 80 percent decrease in pain score which persisted for greater than 40 days.

Figure 20 is a graphical representation of the Ritchie Articular Index, (a scale scored of joint tenderness), is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent decrease in the the Ritchie Articular Index, which persisted for greater than 40 days.

Figure 21 is a graphical representation of the number of swollen joints for the five patients in group I and the four patients in Group II is plotted as the mean percent of baseline value versus time. Both groups showed an approximately 70 to 80 percent decrease in swollen joints, which persisted for 30 to 40 days.

Figure 22 is a graphical representation of the serum C-reactive protein for four to five patients in group I, and three of the for patients in group II, is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 80 percent reduction CRP which persisted for 30 to 40 days. The values for patient number 1 and patient number 7 were omitted from the computations on which the plots are based, since these patients did not have elevated CRP values at baseline.

Figure 23 is a graphical representation of the erythrocyte sedimentation rate for the five patients in group I and three of the patients in group II is plotted as the mean percent of the baseline value versus time. Both groups showed an approximately 40 percent reduction in ESR which persisted for at least 40 days. The data from patient number 9 is omitted from the computations on which the plots were based, since this patient did not have an elevated ESR at baseline.

Figure 24 is a graphical representation of the index of Disease Activity, (a composite score of several parameters of disease activity), for the five patients in group I, and the four patients in group II, is plotted as the mean percent of the baseline value versus time. Both groups showed a clinically significant reduction in IDA, which persisted for at least 40 days.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides anti-tissue necrosis factor (TNF) murine antibodies and chimeric murine-human antibodies, and fragments and regions thereof, which inhibit or neutralize TNF biological activity *in vivo* and are specific for human tumor necrosis factor-alpha (hTNF α), which can be used for diagnostic and therapeutic purposes in subjects having pathologies or conditions associated with the presence of a substance reactive with anti-TNF antibody, in particular hTNF α , in amounts exceeding those present in a normal healthy subject. Antibodies, and fragments, regions and derivatives thereof, of the present invention preferably contain at least one V region which recognizes an epitope of TNF which has inhibiting and/or neutralizing biological activity *in vivo*.

Unexpectedly, mAbs of the present invention can block the action of TNF- α without binding to the putative receptor binding locus as presented by Eck and Sprang (J. Biol. Chem. 264(29), 17595-17605 (1989), as amino acids 11-13, 37-42, 49-57 and 155-157 of hTNF α (of SEQ ID NO:1).

Preferred antibodies of the present invention are murine antibodies or high affinity human-murine chimeric anti-TNF α antibodies, and fragments or regions thereof, that have potent inhibiting and/or neutralizing activity *in vivo* against human TNF α . Such antibodies and chimeric antibodies include those generated by immunization using purified recombinant hTNF α (SEQ ID NO:1) or peptide fragments thereof. Such fragments include epitopes of at least 5 amino acids of residues 87-107, or a combination of both of 59-80 and 87-108 of hTNF α (as these corresponding amino acids of SEQ ID NO:1). Additionally, preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention do not recognize amino acids from at least one of amino acids 11-13, 37-42, 49-57 or 155-157 of hTNF α (of SEQ ID NO:1).

Since circulating concentrations of TNF tend to be extremely low, in the range of about 10 pg/ml in non-septic individuals, and reaching about 50 pg/ml in septic patients and above 100 pg/ml in the sepsis syndrome (Hammerle, A.F. et al., 1989, supra) or may be only be detectable at sites of TNF-mediated pathology, it is preferred to use high affinity and/or potent *in vivo* TNF-inhibiting and/or neutralizing antibodies, fragments or regions thereof, for both TNF immunoassays and therapy of TNF-mediated pathology. Such antibodies, fragments, or regions, will preferably have an affinity for hTNF α , expressed as K_a , of at least $10^8 M^{-1}$, more preferably, at least $10^9 M^{-1}$, such as $5 \times 10^8 M^{-1}$, $8 \times 10^8 M^{-1}$, $2 \times 10^9 M^{-1}$, $4 \times 10^9 M^{-1}$, $6 \times 10^9 M^{-1}$, $8 \times 10^9 M^{-1}$.

Preferred for human therapeutic use are high affinity murine and chimeric antibodies, and fragments, regions and derivatives having potent *in vivo* TNF α -inhibiting and/or neutralizing activity, according to the present invention, that block TNF-induced IL-6 secretion. Also preferred for human therapeutic uses are such high affinity murine and chimeric anti-TNF α antibodies, and fragments, regions and derivatives

thereof, that block TNF-induced procoagulant activity, including blocking of TNF-induced expression of cell adhesion molecules such as ELAM-1 and ICAM-1 and blocking of TNF mitogenic activity, *in vivo*, *in situ*, and *in vitro*.

5 Preferred anti-TNF mAbs are those which will competitively inhibit *in vivo* the binding to human TNF α of anti-TNF α murine mAb A2, chimeric mAb cA2, or an antibody having substantially the same specific binding characteristics, as well as fragments and regions thereof. Preferred antibodies of the
10 present invention are those that bind epitopes recognized by A2 and cA2, which are included in amino acids 59-80 and/or 87-108 of hTNF α (as these corresponding amino acids of SEQ ID NO:1), such that the epitopes consist of at least 5 amino acids which comprise at least one amino acid from the above portions of
15 human TNF α . Preferred methods for determining mAb specificity and affinity by competitive inhibition can be found in Muller, Meth. Enzymol. 92:589-601 (1983), which is entirely incorporated by reference.

As examples of antibodies according to the present
20 invention, murine mAb A2 of the present invention is produced by a cell line designated c134A. Chimeric antibody cA2 is produced by a cell line designated c168A. Cell line c134A is deposited as a research cell bank in the Centocor Cell Biology Services Depository, and cell line c168A(RCB) is deposited as a research
25 cell bank in the Centocor Corporate Cell Culture Research and Development Depository, both at Centocor, 200 Great Valley Parkway, Malvern, Pennsylvania, 19355. The c168A cell line is also deposited at Centocor BV, Leiden, The Netherlands.

Furthermore, c168A was deposited as of the filing date
30 of the present application at the American Type Culture Collection, Rockville, Maryland, as a "Culture Safe Deposit."

The term "epitope" is meant to refer to that portion of any molecule capable of being recognized by and bound by an antibody. Epitopes usually consist of chemically active surface
35 groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics. By "inhibiting and/or neutralizing epitope" is intended an epitope, which, when bound by an antibody, results in loss of biological activity of

the molecule or organism containing the epitope, *in vivo*, *in vitro* and *in situ*, more preferably *in vivo*, including binding of TNF to a TNF receptor. Preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention

5 recognize epitopes including 5 amino acids comprising at least one amino acid from amino acids residues 87-108 or both residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1). Preferred antibodies, fragments and regions of anti-TNF antibodies of the present invention do not recognize epitopes from at least one of

10 amino acids 11-13, 37-42, 49-57 or 155-157 of hTNF α (of SEQ ID NO:1). In a preferred embodiment, the epitope comprises at least 2 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 3 amino acids from

15 residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 4 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 5 amino acids from residues 87-108 or

20 both residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 6 amino acids from residues 87-108 or both residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1). In another preferred embodiment, the epitope comprises at least 7 amino acids from

25 residues 87-108 or both residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1).

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of

30 binding to an epitope of that antigen. An antigen may have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by

35 other antigens. Preferred antigens that bind antibodies, fragments and regions of anti-TNF antibodies of the present invention include at least 5 amino acids comprising at least one of amino acids residues 87-108 or both residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1). Preferred antigens that bind

antibodies, fragments and regions of anti-TNF antibodies of the present invention do not include amino acids of amino acids 11-13, 37-42, 49-57 or 155-157 of hTNF α (SEQ ID NO:1)

The term "antibody" is meant to include a polyclonal, or monoclonal antibody, and fragments and regions thereof, as well as derivatives thereof, which are capable of binding portions of TNF that inhibit binding to TNF receptors by TNF. Fragments include, for example, Fab, Fab', F(ab')₂, and Fv. These fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)). These fragments are produced from intact antibodies using methods well known in the art, for example by proteolytic cleavage with enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments). Regions of anti-TNF antibodies of the present invention include at least one of a heavy chain constant region (H_c), a heavy chain variable region (H_v), a light chain variable region (L_v) and a light chain constant regions (L_c), wherein a polyclonal, monoclonal antibody, fragments and regions thereof include at least one heavy chain variable region (H_v) or light chain variable region (L_v) which binds a portion of a TNF and inhibits TNF biological activity.

In a preferred embodiment, the antibody is a monoclonal antibody which binds amino acids of an epitope of TNF, which antibody is designated A2, rA2 or cA2, which is produced by a hybridoma or by a recombinant host. In another preferred embodiment, the antibody is a chimeric antibody which recognizes an epitope recognized by A2. In a more preferred embodiment, the antibody is a chimeric antibody designated as chimeric A2 (cA2).

The murine and chimeric antibodies, fragments and regions of the present invention comprise individual heavy (H) and light (L) immunoglobulin chains. A chimeric H chain comprises an antigen binding region derived from the H chain of a non-human antibody specific for TNF, which is linked to at least a portion of a human H chain C region (C_H).

A chimeric L chain according to the present invention, comprises an antigen binding region derived from the L chain of

a non-human antibody specific for TNF, linked to at least a portion of a human L chain C region (C_L).

As used herein, the term "antigen binding region" refers to that portion of an antibody molecule which contains the amino acid residues that interact with an antigen and confer on the antibody its specificity and affinity for the antigen. The antibody region includes the "framework" amino acid residues necessary to maintain the proper conformation of the antigen-binding residues.

As used herein, the term "chimeric antibody" includes monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer (HL) formed by a chimeric H chain associated through disulfide bridges with a chimeric L chain. A divalent chimeric antibody is tetramer (H_2L_2) formed by two HL dimers associated through at least one disulfide bridge. A polyvalent chimeric antibody can also be produced, for example, by employing a C_H region that aggregates (e.g., from an IgM H chain, or μ chain).

The invention also provides for "derivatives" of the murine or chimeric antibodies, fragments, regions or derivatives thereof, which term includes those proteins encoded by truncated or modified genes to yield molecular species functionally resembling the immunoglobulin fragments. The modifications include, but are not limited to, addition of genetic sequences coding for cytotoxic proteins such as plant and bacterial toxins. The fragments and derivatives can be produced from any of the hosts of this invention. Alternatively, anti-TNF antibodies, fragments and regions can be bound to cytotoxic proteins or compounds *in vitro*, to provide cytotoxic anti-TNF antibodies which would selectively kill cells having TNF receptors.

Antibodies, fragments or derivatives having chimeric H chains and L chains of the same or different V region binding specificity, can be prepared by appropriate association of the individual polypeptide chains, as taught, for example by Sears et al., Proc. Natl. Acad. Sci. USA 72:353-357 (1975). With this approach, hosts expressing chimeric H chains (or their derivatives) are separately cultured from hosts expressing chimeric L chains (or their derivatives), and the immunoglobulin

chains are separately recovered and then associated.
Alternatively, the hosts can be co-cultured and the chains
allowed to associate spontaneously in the culture medium,
followed by recovery of the assembled immunoglobulin, fragment
or derivative.

The antigen binding region of the chimeric antibody of
the present invention is derived preferably from a non-human
antibody specific for human TNF. Preferred sources for the DNA
encoding such a non-human antibody include cell lines which
produce antibody, preferably hybrid cell lines commonly known as
hybridomas. A preferred hybridoma is the A2 hybridoma cell
line.

The hybrid cells are formed by the fusion of a
non-human anti-hTNF α antibody-producing cell, typically a spleen
cell of an animal immunized against either natural or
recombinant human TNF, or a peptide fragment of the human TNF α
protein sequence. Alternatively, the non-human anti-TNF α
antibody-producing cell may be a B lymphocyte obtained from the
blood, spleen, lymph nodes or other tissue of an animal
immunized with TNF.

The antibody-producing cell contributing the
nucleotide sequences encoding the antigen-binding region of the
chimeric antibody of the present invention may also be produced
by transformation of a non-human, such as a primate, or a human
cell. For example, a B lymphocyte which produces anti-TNF
antibody may be infected and transformed with a virus such as
Epstein-Barr virus to yield an immortal anti-TNF producing cell
(Kozbor et al. Immunol. Today 4:72-79 (1983)). Alternatively,
the B lymphocyte may be transformed by providing a transforming
gene or transforming gene product, as is well-known in the art.

Preferably, the antigen binding region will be of
murine origin. In other embodiments, the antigen binding region
may be derived from other animal species, in particular rodents
such as rabbit, rat or hamster.

The second fusion partner, which provides the
immortalizing function, may be lymphoblastoid cell or a
plasmacytoma or myeloma cell, which is not itself an antibody
producing cell, but is malignant. Preferred fusion partner
cells include the hybridoma SP2/0-Ag14, abbreviated as SP2/0

(ATCC CRL1581) and the myeloma P3X63Ag8 (ATCC TIB9), or its derivatives (see: Hartlow, E. et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988)).

5 Murine hybridomas which produce mAb specific for human TNF α are formed by the fusion of a mouse fusion partner cell, such as SP2/0, and spleen cells from mice immunized against purified hTNF α , recombinant hTNF α , natural or synthetic TNF peptides, including peptides including 5 or more amino acids
10 selected from residues 59-80, and 87-108 of TNF (of SEQ ID NO:1) or other biological preparations containing TNF. To immunize the mice, a variety of different conventional protocols may be followed. For example, mice may receive primary and boosting immunizations of TNF.

15 TNF residues 87-108 or both residues 59-80 and 87-108 of TNF (of SEQ ID NO:1), fragments or combinations of peptides containing therein are useful as immunogens to raise antibodies that will recognize peptide sequences presented in the context of the native TNF molecule.

20 Epitopes recognized by antibodies, and fragments and regions thereof, of the present invention include 5 or more amino acids comprising at least one amino acid of each or both of the following amino acid sequences of TNF, which provide a topographical epitope of TNF which is recognized by, and binds
25 with anti-TNF activity, an antibody, and fragments, and variable regions thereof, of the present invention:

59-80: Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-Gln-Gly-Cys-
Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-Thr-Ile
(AA 59-80 of SEQ ID NO:1); and

30 87-108: Tyr-Gln-Thr-Lys-Val-Asn-Leu-Leu-Ser-Ala-Ile-
Lys-Ser-
Pro-Cys-Gln-Arg-Glu-Thr-Pro-Glu-Gly
(AA 87-108 of SEQ ID NO:1).

35 Particular peptides which can be used to generate antibodies of the present invention include combinations of amino acids selected from at least residues 87-108 or both residues 59-80 and 87-108, which are combined to provide an epitope of TNF that is bound by anti-TNF antibodies, fragments and regions thereof, and which binding provided anti-TNF

biological activity. Such epitopes include at least 1-5 amino acids and less than 22 amino acids from residues 87-108 or each of residues 59-80 and 87-108, which in combination with other amino acids of TNF provide epitopes of at least 5 amino acids in length.

The techniques to raise antibodies of the present invention to small peptide sequences that recognize and bind to those sequences in the free or conjugated form or when presented as a native sequence in the context of a large protein are well known in the art. Such antibodies include murine, murine human and human-human antibodies produced by hybridoma or recombinant techniques known in the art.

The identification of these peptide sequences recognized by mAbs of the present invention provides the information necessary to generate additional monoclonal antibodies with binding characteristics and therapeutic utility that parallel the embodiments of this application.

In a preferred embodiment, the amino acids of the epitope are not of at least one of amino acids 11-13, 37-42, 49-57 and 155-157 of hTNF α (of SEQ ID NO:1).

The cell fusions are accomplished by standard procedures well known to those skilled in the field of immunology (Kohler and Milstein, Nature 256:495-497 (1975) and U.S. Patent No. 4,376,110; Hartlow, E. et al., supra; Campbell, A., "Monoclonal Antibody Technology," In: Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13 (Burdon, R., et al., eds.), Elsevier, Amsterdam (1984); Kennett et al., Monoclonal Antibodies (Kennett et al., eds. pp. 365-367, Plenum Press, NY, 1980); de St. Groth, S.F., et al., J. Immunol. Meth. 35: 1-21 (1980); Galfre, G. et al., Methods Enzymol. 73:3-46 (1981); Goding, J.W. 1987. Monoclonal Antibodies: Principles and Practice. 2nd ed. Academic Press, London, 1987) ;

Fusion partner cell lines and methods for fusing and selecting hybridomas and screening for mAbs are well known in the art (Hartlow, E. et al., supra; Kawamoto, T. et al., Meth. Enzymol 121:266-277 (1986); Kearney, J.F. et al., J. Immunol. 123:1548-1550 (1979); Kilmartin, J.V. et al., J. Cell Biol. 93:576-582 (1982); Kohler, G. et al., Eur. J. Immunol. 6:292-295 (1976); Lane, D.P. et al., J. Immunol. Meth.

47:303-307 (1981); Mueller, U.W. et al., J. Immunol. Meth.
87:193-196 (1986); Pontecorvo, G., Somatic Cell Genet. 1:397-400
(1975); Sharo, J., et al., Proc. Natl. Acad. Sci. USA
76:1420-1424 (1979); Shulman, M. et al., Nature 276:269-270
5 (1978); Springer, T.A. (ed), Hybridoma Technology in the
Biosciences and Medicine, Plenum Press, New York, 1985; and
Taggart, R.T. et al., Science 219:1228-1230 (1982)).

The hTNF α -specific murine or chimeric mAb of the
present invention may be produced in large quantities by
10 injecting hybridoma or transfectoma cells secreting the antibody
into the peritoneal cavity of mice and, after appropriate time,
harvesting the ascites fluid which contains a high titer of the
mAb, and isolating the mAb therefrom. For such in vivo
production of the mAb with a non-murine hybridoma (e.g., rat or
15 human), hybridoma cells are preferably grown in irradiated or
athymic nude mice.

Alternatively, the antibodies may be produced by
culturing hybridoma or transfectoma cells in vitro and isolating
secreted mAb from the cell culture medium.

20 Monoclonal antibodies of the present invention
recognize epitopes including non-contiguous residues located
within the non-contiguous sequences residues 87-108 or both
residues 59-80 and 87-108 of TNF (of SEQ ID NO:1). Preferred
anti-TNF mAbs are those that inhibit this binding of human TNF- α
25 to its receptors by virtue of their ability to bind to one or
more of these peptide sequences. These antibodies would block
the activity of TNF by virtue of binding to the epitope of
sequences including 87-108 and/or 110-128 of TNF (of SEQ ID
NO:1). Such binding is demonstrated to inhibit TNF activity, as
30 described herein. Particular peptides which can be used to
screen antibodies of the present invention include combinations
of amino acids selected from at least residues 87-108 or both
residues 59-80 and 87-108, which are combined to provide an
epitope of TNF that is bound by anti-TNF antibodies, fragments
35 and regions thereof, of the present invention, and which binding
provided anti-TNF biological activity. Such epitopes include at
least 1-5 amino acids and less than 22 amino acids from residues
87-108 or each of residues 59-80 and 87-108, which in

combination with other amino acids of TNF provide epitopes of at least 5 amino acids in length.

Recombinant Expression of Anti-TNF Activity Antibodies

Recombinant murine or chimeric murine-human or human-human antibodies that inhibit TNF and bind an epitope included in the amino acid sequences residues 87-108 or both residues 59-80 and 87-108 of hTNF α (of SEQ ID NO:1), can be provided according to the present invention using known techniques based on the teaching provided herein.

The DNA encoding an anti-TNF antibody of the present invention may be genomic DNA or cDNA which encodes at least one of the heavy chain constant region (H_c), the heavy chain variable region (H_v), the light chain variable region (L_v) and the light chain constant regions (L_c). A convenient alternative to the use of chromosomal gene fragments as the source of DNA encoding the murine V region antigen-binding segment is the use of cDNA for the construction of chimeric immunoglobulin genes, as reported by Liu *et al.* (Proc. Natl. Acad. Sci., USA 84:3439 (1987) and J. Immunology 139:3521 (1987), which references are hereby entirely incorporated by reference. The use of cDNA requires that gene expression elements appropriate for the host cell be combined with the gene in order to achieve synthesis of the desired protein. The use of cDNA sequences is advantageous over genomic sequences (which contain introns), in that cDNA sequences can be expressed in bacteria or other hosts which lack appropriate RNA splicing systems.

For example, a cDNA encoding a murine V region antigen-binding segment having anti-TNF activity can be provided using known methods based on the use of the DNA sequence presented in Figure 17A (SEQ ID NO:2). Alternatively, a cDNA encoding a murine C region antigen-binding segment having anti-TNF activity can be provided using known methods based on the use of the DNA sequence presented in Figure 17B (SEQ ID NO:3). Probes that bind a portion of the DNA sequence presented in Figure 17A or 17B can be used to isolate DNA from hybridomas expressing TNF antibodies, fragments or regions, as presented herein, according to the present invention, by known methods.

Oligonucleotides representing a portion of the variable region presented in Figure 17A or 17B sequence are

useful for screening for the presence of homologous genes and for the cloning of such genes encoding variable or constant regions of an anti-TNF antibody. Such probes preferably bind to portions of sequences according to Figures 17A or 17B which
5 encode light chain or heavy chain variable regions which bind an activity inhibiting epitope of TNF, especially an epitope of at least 5 amino acids of residues 87-108 or a combination of residues 59-80 and 87-108 (of SEQ ID NO:1).

Such techniques for synthesizing such oligonucleotides
10 are well known and disclosed by, for example, Wu, R., et al., Prog. Nucl. Acid. Res. Molec. Biol. 21:101-141 (1978)), and Ausubel et al, eds. Current Protocols in Molecular Biology, Wiley Interscience (1987, 1991), the entire contents of which are herein incorporated by reference.

15 Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid (Watson, J.D. et al., supra). Using the genetic code, one or more different oligonucleotides can be identified, each of which would be capable of encoding the amino acid. The probability
20 that a particular oligonucleotide will, in fact, constitute the actual XXX-encoding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a particular amino acid) in eukaryotic or prokaryotic cells expressing an
25 anti-TNF antibody or fragment. Such "codon usage rules" are disclosed by Lathe, R., et al., J. Molec. Biol. 183:1-12 (1985). Using the "codon usage rules" of Lathe, a single oligonucleotide, or a set of oligonucleotides, that contains a theoretical "most probable" nucleotide sequence capable of
30 encoding anti-TNF variable or constant region sequences is identified.

Although occasionally an amino acid sequence may be encoded by only a single oligonucleotide, frequently the amino acid sequence may be encoded by any of a set of similar
35 oligonucleotides. Importantly, whereas all of the members of this set contain oligonucleotides which are capable of encoding the peptide fragment and, thus, potentially contain the same oligonucleotide sequence as the gene which encodes the peptide fragment, only one member of the set contains the nucleotide

sequence that is identical to the nucleotide sequence of the gene. Because this member is present within the set, and is capable of hybridizing to DNA even in the presence of the other members of the set, it is possible to employ the unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes the protein.

The oligonucleotide, or set of oligonucleotides, containing the theoretical "most probable" sequence capable of encoding an anti-TNF antibody or fragment including a variable or constant region is used to identify the sequence of a complementary oligonucleotide or set of oligonucleotides which is capable of hybridizing to the "most probable" sequence, or set of sequences. An oligonucleotide containing such a complementary sequence can be employed as a probe to identify and isolate the variable or constant region anti-TNF gene (Sambrook et al., supra).

A suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the variable or constant anti-TNF region (or which is complementary to such an oligonucleotide, or set of oligonucleotides) is identified (using the above-described procedure), synthesized, and hybridized by means well known in the art, against a DNA or, more preferably, a cDNA preparation derived from cells which are capable of expressing anti-TNF antibodies or variable or constant regions thereof. Single stranded oligonucleotide molecules complementary to the "most probable" variable or constant anti-TNF region peptide coding sequences can be synthesized using procedures which are well known to those of ordinary skill in the art (Belagaje, R., et al., J. Biol. Chem. 254:5765-5780 (1979); Maniatis, T., et al., In: Molecular Mechanisms in the Control of Gene Expression, Nierlich, D.P., et al., Eds., Acad. Press, NY (1976); Wu, R., et al., Prog. Nucl. Acid Res. Molec. Biol. 21:101-141 (1978); Khorana, R.G., Science 203:614-625 (1979)). Additionally, DNA synthesis may be achieved through the use of automated synthesizers. Techniques of nucleic acid hybridization are disclosed by Sambrook et al. (supra), and by Haymes, B.D., et al. (In: Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, DC

(1985)), which references are herein incorporated by reference. Techniques such as, or similar to, those described above have successfully enabled the cloning of genes for human aldehyde dehydrogenases (Hsu, L.C., et al., Proc. Natl. Acad. Sci. USA 82:3771-3775 (1985)), fibronectin (Suzuki, S., et al., Eur. Mol. Biol. Organ. J. 4:2519-2524 (1985)), the human estrogen receptor gene (Walter, P., et al., Proc. Natl. Acad. Sci. USA 82:7889-7893 (1985)), tissue-type plasminogen activator (Pennica, D., et al., Nature 301:214-221 (1983)) and human term placental alkaline phosphatase complementary DNA (Kam, W., et al., Proc. Natl. Acad. Sci. USA 82:8715-8719 (1985)).

In an alternative way of cloning a polynucleotide encoding an anti-TNF variable or constant region, a library of expression vectors is prepared by cloning DNA or, more preferably, cDNA (from a cell capable of expressing an anti-TNF antibody or variable or constant region) into an expression vector. The library is then screened for members capable of expressing a protein which competitively inhibits the binding of an anti-TNF antibody, such as A2 or cA2, and which has a nucleotide sequence that is capable of encoding polypeptides that have the same amino acid sequence as anti-TNF antibodies or fragments thereof. In this embodiment, DNA, or more preferably cDNA, is extracted and purified from a cell which is capable of expressing an anti-TNF antibody or fragment. The purified cDNA is fragmentized (by shearing, endonuclease digestion, etc.) to produce a pool of DNA or cDNA fragments. DNA or cDNA fragments from this pool are then cloned into an expression vector in order to produce a genomic library of expression vectors whose members each contain a unique cloned DNA or cDNA fragment. Once nucleic acid encoding such variable or constant anti-TNF regions is isolated, the nucleic acid can be appropriately expressed in a host cell, along with other constant or variable heavy or light chain encoding nucleic acid, in order to provide recombinant monoclonal antibodies that bind TNF with inhibitory activity. Such antibodies preferably include a murine anti-TNF variable region which contains a framework residue having complementarity determining residues which are responsible for antigen binding. In a preferred embodiment, an anti-TNF variable light or heavy chain encoded by a nucleic acid as

described above binds an epitope of at least 5 amino acids including residues 87-108 or a combination of residues 59-80 and 87-108 of hTNF (of SEQ ID NO:1).

Human genes which encode the constant (C) regions of the murine and chimeric antibodies, fragments and regions of the present invention can be derived from a human fetal liver library, by known methods. Human C regions genes may be derived from any human cell including those which express and produce human immunoglobulins. The human C_H region can be derived from any of the known classes or isotypes of human H chains, including gamma, μ , α , δ or ϵ , and subtypes thereof, such as G1, G2, G3 and G4. Since the H chain isotype is responsible for the various effector functions of an antibody, the choice of C_H region will be guided by the desired effector functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity (ADCC). Preferably, the C_H region is derived from gamma 1 (IgG1), gamma 3 (IgG3), gamma 4 (IgG4), or μ (IgM).

The human C_L region can be derived from either human L chain isotype, kappa or lambda.

Genes encoding human immunoglobulin C regions are obtained from human cells by standard cloning techniques (Sambrook, J. et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989) and Ausubel et al, eds. Current Protocols in Molecular Biology (1987-1991)). Human C region genes are readily available from known clones containing genes representing the two classes of L chains, the five classes of H chains and subclasses thereof. Chimeric antibody fragments, such as F(ab')₂ and Fab, can be prepared by designing a chimeric H chain gene which is appropriately truncated. For example, a chimeric gene encoding an H chain portion of an F(ab')₂ fragment would include DNA sequences encoding the CH₁ domain and hinge region of the H chain, followed by a translational stop codon to yield the truncated molecule.

Generally, the murine, human or murine and chimeric antibodies, fragments and regions of the present invention are produced by cloning DNA segments encoding the H and L chain antigen-binding regions of a TNF-specific antibody, and joining these DNA segments to DNA segments encoding C_H and C_L regions,

respectively, to produce murine, human or chimeric immunoglobulin-encoding genes.

Thus, in a preferred embodiment, a fused chimeric gene is created which comprises a first DNA segment that encodes at least the antigen-binding region of non-human origin, such as a functionally rearranged V region with joining (J) segment, linked to a second DNA segment encoding at least a part of a human C region.

Therefore, cDNA encoding the antibody V and C regions, the method of producing the chimeric antibody according to the present invention involves several steps, outlined below:

1. isolation of messenger RNA (mRNA) from the cell line producing an anti-TNF antibody and from optional additional antibodies supplying heavy and light constant regions; cloning and cDNA production therefrom;
2. preparation of a full length cDNA library from purified mRNA from which the appropriate V and/or C region gene segments of the L and H chain genes can be: (i) identified with appropriate probes, (ii) sequenced, and (iii) made compatible with a C or V gene segment from another antibody for a chimeric antibody;
3. Construction of complete H or L chain coding sequences by linkage of the cloned specific V region gene segments to cloned C region gene, as described above;
4. Expression and production of L and H chains in selected hosts, including prokaryotic and eukaryotic cells to provide murine-murine, human-murine, human-human or human murine antibodies.

One common feature of all immunoglobulin H and L chain genes and their encoded mRNAs is the J region. H and L chain J regions have different sequences, but a high degree of sequence homology exists (greater than 80%) among each group, especially near the C region. This homology is exploited in this method and consensus sequences of H and L chain J regions may be used to design oligonucleotides for use as primers for introducing useful restriction sites into the J region for subsequent linkage of V region segments to human C region segments.

C region cDNA vectors prepared from human cells can be modified by site-directed mutagenesis to place a restriction site at the analogous position in the human sequence. For example, one can clone the complete human kappa chain C (C_k) region and the complete human gamma-1 C region ($C_{\text{gamma-1}}$). In this case, the alternative method based upon genomic C region clones as the source for C region vectors would not allow these genes to be expressed in bacterial systems where enzymes needed to remove intervening sequences are absent. Cloned V region segments are excised and ligated to L or H chain C region vectors. Alternatively, the human $C_{\text{gamma-1}}$ region can be modified by introducing a termination codon thereby generating a gene sequence which encodes the H chain portion of an Fab molecule. The coding sequences with linked V and C regions are then transferred into appropriate expression vehicles for expression in appropriate hosts, prokaryotic or eukaryotic.

Two coding DNA sequences are said to be "operably linked" if the linkage results in a continuously translatable sequence without alteration or interruption of the triplet reading frame. A DNA coding sequence is operably linked to a gene expression element if the linkage results in the proper function of that gene expression element to result in expression of the coding sequence.

Expression vehicles include plasmids or other vectors. Preferred among these are vehicles carrying a functionally complete human C_H or C_L chain sequence having appropriate restriction sites engineered so that any V_H or V_L chain sequence with appropriate cohesive ends can be easily inserted therein. Human C_H or C_L chain sequence-containing vehicles thus serve as intermediates for the expression of any desired complete H or L chain in any appropriate host.

A chimeric antibody, such as a mouse-human or human-human, will typically be synthesized from genes driven by the chromosomal gene promoters native to the mouse H and L chain V regions used in the constructs; splicing usually occurs between the splice donor site in the mouse J region and the splice acceptor site preceding the human C region and also at the splice regions that occur within the human C_H region;

polyadenylation and transcription termination occur at native chromosomal sites downstream of the human coding regions.

Gene expression elements useful for the expression of cDNA genes include: (a) viral transcription promoters and their enhancer elements, such as the SV40 early promoter (Okayama, H. et al., Mol. Cell. Biol. 3:280 (1983)), Rous sarcoma virus LTR (Gorman, C. et al., Proc. Natl. Acad. Sci., USA 79:6777 (1982)), and Moloney murine leukemia virus LTR (Grosschedl, R. et al., Cell 41:885 (1985)); (b) splice regions and polyadenylation sites such as those derived from the SV40 late region (Okayama et al., supra); and (c) polyadenylation sites such as in SV40 (Okayama et al., supra).

Immunoglobulin cDNA genes may be expressed as described by Liu et al., supra, and Weidle et al., Gene 51:21 (1987), using as expression elements the SV40 early promoter and its enhancer, the mouse immunoglobulin H chain promoter enhancers, SV40 late region mRNA splicing, rabbit β -globin intervening sequence, immunoglobulin and rabbit β -globin polyadenylation sites, and SV40 polyadenylation elements. For immunoglobulin genes comprised of part cDNA, part genomic DNA (Whittle et al., Protein Engineering 1:499 (1987)), the transcriptional promoter is human cytomegalovirus, the promoter enhancers are cytomegalovirus and mouse/human immunoglobulin, and mRNA splicing and polyadenylation regions are from the native chromosomal immunoglobulin sequences.

In one embodiment, for expression of cDNA genes in rodent cells, the transcriptional promoter is a viral LTR sequence, the transcriptional promoter enhancers are either or both the mouse immunoglobulin heavy chain enhancer and the viral LTR enhancer, the splice region contains an intron of greater than 31 bp, and the polyadenylation and transcription termination regions are derived from the native chromosomal sequence corresponding to the immunoglobulin chain being synthesized. In other embodiments, cDNA sequences encoding other proteins are combined with the above-recited expression elements to achieve expression of the proteins in mammalian cells.

Each fused gene is assembled in, or inserted into, an expression vector. Recipient cells capable of expressing the

chimeric immunoglobulin chain gene product are then transfected singly with a chimeric H or chimeric L chain-encoding gene, or are co-transfected with a chimeric H and a chimeric L chain gene. The transfected recipient cells are cultured under
5 conditions that permit expression of the incorporated genes and the expressed immunoglobulin chains or intact antibodies or fragments are recovered from the culture.

In one embodiment, the fused genes encoding the chimeric H and L chains, or portions thereof, are assembled in
10 separate expression vectors that are then used to co-transfect a recipient cell.

Each vector may contain two selectable genes, a first selectable gene designed for selection in a bacterial system and a second selectable gene designed for selection in a eukaryotic
15 system, wherein each vector has a different pair of genes. This strategy results in vectors which first direct the production, and permit amplification, of the fused genes in a bacterial system. The genes so produced and amplified in a bacterial host are subsequently used to co-transfect a eukaryotic cell, and
20 allow selection of a co-transfected cell carrying the desired transfected genes.

Examples of selectable genes for use in a bacterial system are the gene that confers resistance to ampicillin and the gene that confers resistance to chloramphenicol. Preferred
25 selectable genes for use in eukaryotic transfectants include the xanthine guanine phosphoribosyl transferase gene (designated gpt) and the phosphotransferase gene from Tn5 (designated neo).

Selection of cells expressing gpt is based on the fact that the enzyme encoded by this gene utilizes xanthine as a
30 substrate for purine nucleotide synthesis, whereas the analogous endogenous enzyme cannot. In a medium containing (1) mycophenolic acid, which blocks the conversion of inosine monophosphate to xanthine monophosphate, and (2) xanthine, only cells expressing the gpt gene can survive. The product of the
35 neo blocks the inhibition of protein synthesis by the antibiotic G418 and other antibiotics of the neomycin class.

The two selection procedures can be used simultaneously or sequentially to select for the expression of immunoglobulin chain genes introduced on two different DNA

vectors into a eukaryotic cell. It is not necessary to include different selectable markers for eukaryotic cells; an H and an L chain vector, each containing the same selectable marker can be co-transfected. After selection of the appropriately resistant cells, the majority of the clones will contain integrated copies of both H and L chain vectors.

Alternatively, the fused genes encoding the chimeric H and L chains can be assembled on the same expression vector.

For transfection of the expression vectors and production of the chimeric antibody, the preferred recipient cell line is a myeloma cell. Myeloma cells can synthesize, assemble and secrete immunoglobulins encoded by transfected immunoglobulin genes and possess the mechanism for glycosylation of the immunoglobulin. A particularly preferred recipient cell is the Ig-non-producing myeloma cell SP2/0 (ATCC #CRL 8287). SP2/0 cells produce only immunoglobulin encoded by the transfected genes. Myeloma cells can be grown in culture or in the peritoneal cavity of a mouse, where secreted immunoglobulin can be obtained from ascites fluid. Other suitable recipient cells include lymphoid cells such as B lymphocytes of human or non-human origin, hybridoma cells of human or non-human origin, or interspecies heterohybridoma cells.

The expression vector carrying a chimeric antibody construct of the present invention may be introduced into an appropriate host cell by any of a variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium phosphate-precipitation, and application with polycations such as diethylaminoethyl (DEAE) dextran, and such mechanical means as electroporation, direct microinjection, and microprojectile bombardment (Johnston *et al.*, Science 240:1538 (1988)). A preferred way of introducing DNA into lymphoid cells is by electroporation (Potter *et al.*, Proc. Natl. Acad. Sci. USA 81:7161 (1984); Yoshikawa, K. *et al.*, Jpn. J. Cancer Res. 77:1122-1133). In this procedure, recipient cells are subjected to an electric pulse in the presence of the DNA to be incorporated. Typically, after transfection, cells are allowed to recover in complete medium for about 24 hours, and are then seeded in 96-well culture plates in the presence of the

selective medium. G418 selection is performed using about 0.4 to 0.8 mg/ml G418. Mycophenolic acid selection utilizes about 6 μ g/ml plus about 0.25 mg/ml xanthine. The electroporation technique is expected to yield transfection frequencies of about 10⁻⁵ to about 10⁻⁴ for Sp2/0 cells. In the protoplast fusion method, lysozyme is used to strip cell walls from catarrhal harboring the recombinant plasmid containing the chimeric antibody gene. The resulting spheroplasts are fused with myeloma cells with polyethylene glycol. The immunoglobulin genes of the present invention can also be expressed in nonlymphoid mammalian cells or in other eukaryotic cells, such as yeast, or in prokaryotic cells, in particular bacteria.

Yeast provides substantial advantages over bacteria for the production of immunoglobulin H and L chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy number plasmids which can be used for production of the desired proteins in yeast. Yeast recognizes leader sequences of cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides) (Hitzman, et al., 11th International Conference on Yeast, Genetics and Molecular Biology, Montpellier, France, September 13-17, 1982).

Yeast gene expression systems can be routinely evaluated for the levels of production, secretion and the stability of chimeric H and L chain proteins and assembled murine and chimeric antibodies, fragments and regions. Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeasts are grown in media rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase (PGK) gene can be utilized. A number of approaches may be taken for evaluating optimal expression plasmids for the expression of cloned immunoglobulin cDNAs in yeast (see Glover, D.M., ed., DNA Cloning, Vol. II, pp45-66, IRL Press, 1985).

Bacterial strains may also be utilized as hosts for the production of antibody molecules or antibody fragments described by this invention, E. coli K12 strains such as E. coli W3110 (ATCC 27325), and other enterobacteria such as Salmonella typhimurium or Serratia marcescens, and various Pseudomonas species may be used.

Plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these bacterial hosts. The vector carries a replication site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. A number of approaches may be taken for evaluating the expression plasmids for the production of murine and chimeric antibodies, fragments and regions or antibody chains encoded by the cloned immunoglobulin cDNAs in bacteria (see Glover, D.M., ed., DNA Cloning, Vol. I, IRL Press, 1985).

Preferred hosts are mammalian cells, grown in vitro or in vivo. Mammalian cells provide post-translational modifications to immunoglobulin protein molecules including leader peptide removal, folding and assembly of H and L chains, glycosylation of the antibody molecules, and secretion of functional antibody protein.

Mammalian cells which may be useful as hosts for the production of antibody proteins, in addition to the cells of lymphoid origin described above, include cells of fibroblast origin, such as Vero (ATCC CRL 81) or CHO-K1 (ATCC CRL 61).

Many vector systems are available for the expression of cloned H and L chain genes in mammalian cells (see Glover, D.M., ed., DNA Cloning, Vol. II, pp143-238, IRL Press, 1985). Different approaches can be followed to obtain complete H₂L₂ antibodies. As discussed above, it is possible to co-express H and L chains in the same cells to achieve intracellular association and linkage of H and L chains into complete tetrameric H₂L₂ antibodies. The co-expression can occur by using either the same or different plasmids in the same host. Genes for both H and L chains can be placed into the same plasmid, which is then transfected into cells, thereby selecting directly for cells that express both chains. Alternatively, cells may be transfected first with a plasmid encoding one chain, for example

the L chain, followed by transfection of the resulting cell line with an H chain plasmid containing a second selectable marker. Cell lines producing H₂L₂ molecules via either route could be transfected with plasmids encoding additional copies of H, L, or H plus L chains in conjunction with additional selectable markers to generate cell lines with enhanced properties, such as higher production of assembled H₂L₂ antibody molecules or enhanced stability of the transfected cell lines.

In addition to monoclonal or chimeric anti-TNF antibodies, the present invention is also directed to an anti-idiotypic (anti-Id) antibody specific for the anti-TNF antibody of the invention. An anti-Id antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding region of another antibody. The antibody specific for TNF is termed the idiotypic or Id antibody. The anti-Id can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the Id antibody with the Id antibody or the antigen-binding region thereof. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody and produce an anti-Id antibody. The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original antibody which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against TNF according to the present invention may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice can be used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for a TNF epitope.

The antibodies, fragments and derivatives of the present invention are useful for treating a subject having a

pathology or condition associated with levels of a substance reactive with an anti-TNF antibody, in particular TNF, in excess of the levels present in a normal healthy subject. Such pathologies include, but are not limited to, sepsis syndrome, including cachexia, circulatory collapse and shock resulting from acute or chronic bacterial infection, acute and chronic parasitic or infectious processes, including bacterial, viral and fungal infections, acute and chronic immune and autoimmune pathologies, such as systemic lupus erythematosus and rheumatoid arthritis, alcohol-induced hepatitis, chronic inflammatory pathologies such as sarcoidosis and Crohn's pathology, vascular inflammatory pathologies such as disseminated intravascular coagulation, graft-versus-host pathology, Kawasaki's pathology; malignant pathologies involving TNF-secreting tumors and neurodegenerative diseases.)

Neurodegenerative diseases include, but are not limited to, AIDS dementia complex, demyelinating diseases, such as multiple sclerosis and acute transverse myelitis; extrapyramidal and cerebellar disorders' such as lesions of the corticospinal system; disorders of the basal ganglia or cerebellar disorders; hyperkinetic movement disorders such as Huntington's Chorea and senile chorea; drug-induced movement disorders, such as those induced by drugs which block CNS dopamine receptors; hypokinetic movement disorders, such as Parkinson's disease; Progressive supranucleo Palsy; structural lesions of the cerebellum; spinocerebellar degenerations, such as spinal ataxia, Friedreich's ataxia, cerebellar cortical degenerations, multiple systems degenerations (Mencel, Dejerine-Thomas, Shi-Drager, and Machado-Joseph); systemic disorders (Refsum's disease, abetalipoproteinemia, ataxia, telangiectasia, and mitochondrial multi.system disorder); demyelinating core disorders, such as multiple sclerosis, acute transverse myelitis; and disorders of the motor unit' such as neurogenic muscular atrophies (anterior horn cell degeneration, such as amyotrophic lateral sclerosis, infantile spinal muscular atrophy and juvenile spinal muscular atrophy); Alzheimer's disease; Down's Syndrome in middle age; Diffuse Lewy body disease; Senile Dementia of Lewy body type; Wernicke-Korsakoff syndrome; chronic alcoholism; Creutzfeldt-Jakob disease; Subacute sclerosing

panencephalitis Hallerrorden-Spatz disease; and Dementia pugilistica. One preferred neurodegenerative disease is multiple sclerosis. See, e.g., Berkow et al, eds., The Merck Manual, 15th edition, Merck and Co., Rahway, N.J., 1987, which
5 reference, and references cited therein, are entirely incorporated herein by reference.

Such treatment comprises parenterally administering a single or multiple doses of the antibody, fragment or derivative. Preferred for human pharmaceutical use are high
10 affinity potent hTNF α -inhibiting and/or neutralizing murine and chimeric antibodies, fragments and regions of this invention.

Monoclonal antibodies of the present invention may be administered by any means that enables the active agent to reach the agent's site of action in the body of a mammal. In the case
15 of the antibodies of this invention, the primary focus is the ability to reach and bind with TNF released by monocytes and macrophages. Because proteins are subject to being digested when administered orally, parenteral administration, i.e., intravenous, subcutaneous, intramuscular, would ordinarily be
20 used to optimize absorption.

Monoclonal antibodies of the present invention may be administered either as individual therapeutic agents or in combination with other therapeutic agents. They can be administered alone, but are generally administered with a
25 pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage administered will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular agent, and its mode and route
30 of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired. Usually a daily dosage of active ingredient can be about 0.1 to 100 milligrams per kilogram of body weight. Ordinarily 0.5 to 50, and
35 preferably 1 to 10 milligrams per kilogram per day given in divided doses 1 to 6 times a day or in sustained release form is effective to obtain desired results.

As a non-limiting example, treatment of rheumatoid arthritis in humans or animals can be provided as a daily dosage

of anti-TNF monoclonal chimeric or murine antibodies of the present invention 0.1 to 100 mg/kg, such as 0.5, 0.9, 1.0, 1.1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 45, 50, 60, 70, 80, 90 or 100 mg/kg, per day, on at least one of day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40, or alternatively, at least one of week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, or any combination thereof, using single or divided doses of every 24, 12, 8, 6, 4, or 2 hours, or any combination thereof.

Dosage forms (composition) suitable for internal administration generally contain from about 1 milligram to about 500 milligrams of active ingredient per unit. In these pharmaceutical compositions the active ingredient will ordinarily be present in an amount of about 0.5-95% by weight based on the total weight of the composition.

For parenteral administration, the antibody can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils may also be used. The vehicle or lyophilized powder may contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques.

Suitable pharmaceutical carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, A. Osol, a standard reference text in this field.

For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

The antibodies of this invention can be adapted for therapeutic efficacy by virtue of their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) against cells having TNF associated with their surface. For these activities, either an

endogenous source or an exogenous source of effector cells (for ADCC) or complement components (for CDC) can be utilized. The murine and chimeric antibodies, fragments and regions of this invention, their fragments, and derivatives can be used
5 therapeutically as immunoconjugates (see for review: Dillman, R.O., Ann. Int. Med. 111:592-603 (1989)). They can be coupled to cytotoxic proteins, including, but not limited to Ricin-A, Pseudomonas toxin, Diphtheria toxin, and TNF. Toxins conjugated to antibodies or other ligands, are known in the art (see, for
10 example, Olsnes, S. et al., Immunol. Today 10:291-295 (1989)). Plant and bacterial toxins typically kill cells by disrupting the protein synthetic machinery.

The antibodies of this invention can be conjugated to additional types of therapeutic moieties including, but not
15 limited to, radionuclides, cytotoxic agents and drugs. Examples of radionuclides which can be coupled to antibodies and delivered in vivo to sites of antigen include ^{212}Bi , ^{131}I , ^{186}Re , and ^{90}Y , which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally
20 irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to antibodies and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and
25 Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a fuller exposition of these classes of drugs which are known in the art, and their mechanisms of action, see Goodman, A.G., et al., Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF
30 THERAPEUTICS, 7th Ed., Macmillan Publishing Co., 1985.

The antibodies of this invention may be advantageously utilized in combination with other monoclonal or murine and chimeric antibodies, fragments and regions, or with lymphokines or hemopoietic growth factors, etc., which serve to increase the
35 number or activity of effector cells which interact with the antibodies.

The antibodies, fragments or derivatives of this invention may also be used in combination with TNF therapy to block undesired side effects of TNF. Recent approaches to

cancer therapy have included direct administration of TNF to cancer patients or immunotherapy of cancer patients with lymphokine activated killer (LAK) cells (Rosenberg et al., New Eng. J. Med. 313:1485-1492 (1985)) or tumor infiltrating lymphocytes (TIL) (Kurnick et al. (Clin. Immunol. Immunopath. 38:367-380 (1986); Kradin et al., Cancer Immunol. Immunother. 24:76-85 (1987); Kradin et al., Transplant. Proc. 20:336-338 (1988)). Trials are currently underway using modified LAK cells or TIL which have been transfected with the TNF gene to produce large amounts of TNF. Such therapeutic approaches are likely to be associated with a number of undesired side effects caused by the pleiotropic actions of TNF which were described above. According to the present invention, these side effects can be reduced by concurrent treatment of a subject receiving TNF or cells producing large amounts of TIL with the antibodies, fragments or derivatives of the present invention. Effective doses are as described above. The dose level will require adjustment according to the dose of TNF or TNF-producing cells administered, in order to block side effects without blocking the main anti-tumor effect of TNF. One of ordinary skill in the art will know how to determine such doses without undue experimentation.

The murine and chimeric antibodies, fragments and regions, fragments, or derivatives of this invention, attached to a solid support, can be used to remove TNF from fluids or tissue or cell extracts. In a preferred embodiment, they are used to remove TNF from blood or blood plasma products. In another preferred embodiment, the murine and chimeric antibodies, fragments and regions are advantageously used in extracorporeal immunoabsorbent devices, which are known in the art (see, for example, Seminars in Hematology, Vol. 26 (2 Suppl. 1) (1989)). Patient blood or other body fluid is exposed to the attached antibody, resulting in partial or complete removal of circulating TNF (free or in immune complexes), following which the fluid is returned to the body. This immunoabsorption can be implemented in a continuous flow arrangement, with or without interposing a cell centrifugation step. See, for example, Terman, D.S. et al., J. Immunol. 117:1971-1975 (1976).

Treatment of Arthritis, Sepsis, Allograft Rejection and Graft Versus Host Disease. In rheumatoid arthritis, the main presenting symptoms are pain, stiffness, swelling, and loss of function (Bennett JC. The etiology of rheumatoid arthritis.

5 In Textbook of Rheumatology (Kelley WN, Harris ED, Ruddy S, Sledge CB, eds.) WB Saunders, Philadelphia pp 879-886, 1985). The multitude of drugs used in controlling such symptoms seems largely to reflect the fact that none is ideal. Although there
10 have been many years of intense research into the biochemical, genetic, microbiological, and immunological aspects of rheumatoid arthritis, its pathogenesis is not completely understood, and none of the treatments clearly stop progression of joint destruction (Harris ED. Rheumatoid Arthritis: The clinical spectrum. In Textbook of Rheumatology (Kelley WN,
15 Harris ED, Ruddy S, Sledge CB, eds.) WB Saunders, Philadelphia pp 915-990, 1985).

TNF α is of major importance in the pathogenesis of rheumatoid arthritis. TNF α is present in rheumatoid arthritis joint tissues and synovial fluid at the protein and mRNA level
20 (Buchan G, Barrett K, Turner M, Chantry D, Maini RN, and Feldmann M. Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 α . Clin. Exp. Immunol 73: 449-455, 1988), indicating local synthesis. However detecting TNF α in rheumatoid arthritis
25 joints even in quantities sufficient for bioactivation does not necessarily indicate that it is important in the pathogenesis of rheumatoid arthritis, nor that it is a good candidate therapeutic target. In order to address these questions, the effects of anti-TNF antibody (rabbit or monoclonal) on
30 rheumatoid joint cell cultures, and for comparison, osteoarthritic cell cultures, have been studied. The initial result, that IL-1 production was abolished suggested that TNF α was a therapeutic target for the therapy of rheumatoid arthritis, since anti-TNF α would block both TNF and IL-1, the
35 two cytokines known to be involved in cartilage and bone destruction (Brennan FM, Chantry D, Jackson A, Maini RN, and Feldmann M. Inhibitory effect of TNF α antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. Lancet II: 244-247, 1989).

Subsequent studies in rheumatoid arthritis tissues have supported this hypothesis. Thus it was found that anti-TNF abrogated the production of another proinflammatory cytokine, GM-CSF (Haworth C, Brennan FM, Chantry D, Maini RN, and Feldmann M. GM-CSF expression in rheumatoid arthritis: regulation by tumour necrosis factor alpha. Eur. J. Immunol. 21:2575-2579, 1991). This observation has been independently confirmed (Alvaro-Gracia JM, Zvaifler NJ, Brown CB, Caushansky K, and Firestein GS. Cytokines in chronic inflammatory arthritis. VI. Analysis of synovial cell involved in granulocyte-macrophage colony-stimulating factor production and gene expression in rheumatoid arthritis and its regulation by IL-1 and tumour necrosis factor- α . 1991). It has also been demonstrated that anti-TNF diminishes cell adhesion and HLA class II expression in rheumatoid arthritis joint cell cultures.

These human studies suggest that blocking TNF would be beneficial in rheumatoid arthritis and have led to experiments that verify this concept in an animal model of arthritis. The collagen type II induced arthritis model in DBA/1 mice has been used and it has been found that monoclonal (hamster) anti murine TNF α or rabbit anti murine TNF α has beneficial effects, if injected weekly from the beginning of collagen injections, or even if injected 21 days after the beginning of collagen injections, just prior to onset or acute arthritis. There was a partial reduction in joint swelling, but more importantly a dramatic reduction in joint destruction and fibrosis. Subsequently it was demonstrated that in rheumatoid arthritis there is augmentation of the endogenous TNF inhibitory system, the soluble TNF receptors (sTNF-R). This system acts to partially inhibit TNF activity in rheumatoid arthritis. Despite considerable upregulation of sTNF-R activity in rheumatoid arthritis joints (and serum) there is still bioactive TNF and so disease activity. This is in contrast to osteoarthritis, where in many samples, there is excess TNF inhibitory activity, and even exogenous TNF can be neutralized.

In order to investigate whether the beneficial effects observed in mice can also be obtained in patients, a chimeric anti-human TNF monoclonal antibody was used in patients with rheumatoid arthritis.

Prior Studies with ANti-TNF Monoclonal Antibody in Humans

In a phase I study, fourteen patients with severe septic shock were administered a neutralizing murine anti-TNF monoclonal antibody in a single dose from 0.4-10 mg/kg (Exley AR, Cohen J, Buurman W, Owen R, Lumley J, Hanson G, Aulakh JM, Bodmer M, Stephens S, Riddell A, Perry M. Monoclonal antibody to TNF in severe septic shock. Lancet II: 1275-1277, 1990). There were no adverse hematological, biochemical or clinical changes associated with the treatment. Seven of the fourteen patients developed a human anti-murine antibody (HAMA) response to treatment.

In another trial, fourteen kidney allograft recipients undergoing prophylactic OKT3 therapy were administered a murine anti-TNF monoclonal antibody in a single dose of either 0.4 mg/kg or 2 mg/kg, for the prevention of the OKT3-induced acute clinical syndrome, known as the cytokine release syndrome. Compared to historical controls the acute OKT3-induce syndrome was reduced into a mild flu-like reaction. The antibody was well tolerated and none of the patients showed an immune response against the anti-TNF antibody.

Administration of another murine anti-TNF monoclonal antibody to 10 patients suffering from severe graft versus host disease has also been reported (Herve P, Wijdenes J, Tiberghien P, Bordigoni P, Racadot E, Wilmer E, Plouvier A, Holler E, Stephan JL, Cahn JY, Roche C. Monoclonal TNF α antibody for the treatment of severe acute graft versus host disease in humans. Lymphoma Res 9: 591, 1990). While the results of this small study were too preliminary for efficacy interpretation, no acute side effects of the treatment were noted.

Rationale for Choice of Chimeric A2 (cA2) Anti-TNF Antibody

Since murine monoclonal antibodies may have properties that are undesirable for human therapeutic use, such as a short free circulating serum half-life and the stimulation of a human anti-murine antibody (HAMA) response, a murine-human chimeric anti-human TNF α monoclonal antibody was developed with high affinity, epitope specificity and the ability to neutralize the cytotoxic effect of human TNF. Chimeric A2 anti-TNF consists of the antigen binding variable region of the high-affinity neutralizing mouse anti-human TNF IgG1 antibody, designated A2,

and the constant regions of a human IgG1, kappa immunoglobulin. The human IgG1 Fc region is expected to: improve allogeneic antibody effector function; increase the circulating serum half-life; and decrease the immunogenicity of the antibody. A
5 similar murine-human chimeric antibody (chimeric 17-1A) has been shown in clinical studies to have a 6-fold longer in vivo circulation time and to be significantly less immunogenic than its corresponding murine monoclonal antibody counterpart (LoBuglio AF, Wheeler RH, Trang dJ, et al. Mouse/human chimeric
10 monoclonal antibody in man: kinetics and immune response. Proc Natl Acad Sci USA 86: 4220-4224, 1988).

The avidity and epitope specificity of the chimeric A2 is derived from the variable region of the murine A2. In a solid phase ELISA, cross-competition for TNF was observed
15 between chimeric and murine A2, indicating an identical epitope specificity of cA2 and murine A2. The specificity of cA2 for TNF- α was confirmed by its inability to neutralize the cytotoxic effects of lymphotoxin(TNF- β). Chimeric A2 neutralizes the cytotoxic effect of both natural and recombinant human TNF in a
20 dose dependent manner. From binding assays of cA2 and recombinant human TNF, the affinity constant of cA2 was calculated to be $1.8 \times 10^9 M^{-1}$.

It has been reported that there are multiple epitopes on TNF to which neutralizing monoclonal antibodies will
25 bind (Moller A, Emling F, Blohm D, Schlick E, Schollmeier K. Monoclonal antibodies to human tumor necrosis factor α : in vitro and in vivo application. Cytokine 2:162-169, 1990). Certain neutralizing epitopes on human TNF are conserved amongst different species. However, the epitope specificity of A2
30 appears to be for an epitope which is restricted to humans and chimpanzees. Therefore, the chimpanzee was chosen as the relevant animal species to determine the toxicological potential and provide safety information for cA2. Chimpanzees were dosed at levels of 15 mg/kg for four to five consecutive days and 30
35 mg/kg once or for three consecutive days. No adverse clinical signs, and no changes considered cA2 treatment related were observed in the monitored parameters including routine hematology and chemistry. Thus, doses of up to 30 mg/kg for three consecutive days were well tolerated in chimpanzees.

Clinical experience with chimeric A2 anti-TNF antibody

The chimeric anti-TNF monoclonal antibody cA2 has been used in two phase I/II studies. In a phase I/II study in septic patients, 20 patients with the sepsis syndrome received a single dose of either 0.1, 1.0, 5.0 or 10 milligrams of cA2 per kilogram bodyweight. Another 60 patients received 100 milligrams of HA-1A, a human anti-lipid A Mab currently under evaluation for gram negative sepsis, followed with either placebo or 1.0, 5.0, or 10 milligrams cA2 per kilogram bodyweight. The cA2 was administered as a single, intravenous infusion over a 60 minute period. Clinical assessment, vital signs, and laboratory parameters were measured before, during and periodically for 28 days after the infusion. In this study, cA2 was well tolerated. No adverse events were reported as "probably" or "definitely" related to cA2. All deaths were reported as "definitely not" related to cA2.

Accordingly, human treatment of rheumatoid arthritis in human patients was expected, and found, to provide a suitable treatment, as described herein.

Diagnostic Methods

The present invention also provides the above antibodies, fragments and derivatives, detectably labeled, as described below, for use in diagnostic methods for detecting TNF α in patients known to be or suspected of having a TNF α -mediated condition.

The antibodies of the present invention are useful for immunoassays which detect or quantitate TNF, or anti-TNF antibodies, in a sample. An immunoassay for TNF typically comprises incubating a biological sample in the presence of a detectably labeled high affinity antibody of the present invention capable of selectively binding to TNF, and detecting the labeled antibody which is bound in a sample. Various clinical immunoassay procedures are described in Immunoassays for the 80's, A. Voller et al., eds., University Park, 1981.

Thus, in this aspect of the invention, the antibody or a biological sample may be added to nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably

labeled TNF-specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on said solid support may then be detected by conventional means.

5 By "solid phase support" or "carrier" is intended any support capable of binding antigen or antibodies. Well-known supports or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified
10 celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to TNF or an anti-TNF antibody. Thus, the support
15 configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers
20 for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-TNF antibody may be determined according to well known methods. Those skilled in the art will be able to determine operative and
25 optimal assay conditions for each determination by employing routine experimentation.

One of the ways in which the TNF-specific antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA), or enzyme-linked
30 immunosorbent assay (ELISA). This enzyme, when subsequently exposed to its substrate, will react with the substrate generating a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes
35 which can be used to detectably label the TNF-specific antibodies of the present invention include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate

isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

5 By radioactively labeling the TNF-specific antibodies, it is possible to detect TNF through the use of a radioimmunoassay (RIA) (see, for example, Work, T.S., et al., Laboratory Techniques and Biochemistry in Molecular Biology, North Holland Publishing Company, N.Y. (1978). The radioactive
10 isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are: ^3H , ^{125}I , ^{131}I , ^{35}S , ^{14}C , and, preferably, ^{125}I .

15 It is also possible to label the TNF-specific antibodies with a fluorescent compound. When the fluorescent labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are
20 fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The TNF-specific antibodies can also be detectably labeled using fluorescence-emitting metals such as ^{152}Eu , or others of the lanthanide series. These metals can be attached
25 to the TNF-specific antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediamine-tetraacetic acid (EDTA).

The TNF-specific antibodies also can be detectably labeled by coupling to a chemiluminescent compound. The
30 presence of the chemiluminescently labeled antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.
35

Likewise, a bioluminescent compound may be used to label the TNF-specific antibody, fragment or derivative of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a

catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin,
5 luciferase and aequorin.

Detection of the TNF-specific antibody, fragment or derivative may be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent
10 material. In the case of an enzyme label, the detection can be accomplished by colorimetric methods which employ a substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

For the purposes of the present invention, the TNF which is detected by the above assays may be present in a biological sample. Any sample containing TNF can be used. Preferably, the sample is a biological fluid such as, for example, blood, serum, lymph, urine, inflammatory exudate,
20 cerebrospinal fluid, amniotic fluid, a tissue extract or homogenate, and the like. However, the invention is not limited to assays using only these samples, it being possible for one of ordinary skill in the art to determine suitable conditions which allow the use of other samples.

25 , In situ detection may be accomplished by removing a histological specimen from a patient, and providing the combination of labeled antibodies of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or
30 fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of TNF but also the distribution of TNF in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological
35 methods (such as staining procedures) can be modified in order to achieve such in situ detection.

The antibody, fragment or derivative of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a

typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support that is insoluble in the fluid being tested and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the TNF from the sample by formation of a binary solid phase antibody-TNF complex. After a suitable incubation period, the solid support is washed to remove the residue of the fluid sample, including unreacted TNF, if any, and then contacted with the solution containing a known quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the TNF bound to the solid support through the unlabeled antibody, the solid support is washed a second time to remove the unreacted labeled antibody. This type of forward sandwich assay may be a simple "yes/no" assay to determine whether TNF is present or may be made quantitative by comparing the measure of labeled antibody with that obtained for a standard sample containing known quantities of TNF. Such "two-site" or "sandwich" assays are described by Wide (Radioimmune Assay Method, Kirkham, ed., E. & S. Livingstone, Edinburgh, 1970, pp. 199-206).

Other type of "sandwich" assays, which may also be useful with TNF, are the so-called "simultaneous" and "reverse" assays. A simultaneous assay involves a single incubation step wherein the antibody bound to the solid support and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support after a

suitable incubation period, is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays. In one embodiment, a combination of antibodies of the present invention specific for separate epitopes may be used to construct a sensitive three-site immunoradiometric assay.

Having now generally described the invention, the same will be further understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE I

Production a Mouse Anti-Human TNF mAb

To facilitate clinical study of TNF mAb a high-affinity potent inhibiting and/or neutralizing mouse anti-human TNF IgG1 mAb designated A2 was produced.

Female BALB/c mice, 10 weeks old, were obtained from the Jackson Laboratory (Bar Harbor, ME). Forty μ g of purified E. coli-derived recombinant human TNF (rhTNF) emulsified with an equal volume of complete Freund's adjuvant (obtained from Difco Laboratories) in 0.4 ml was injected subcutaneously and intraperitoneally (i.p.) into a mouse. One week later, an injection of 5 μ g of rhTNF in incomplete Freund's adjuvant was given i.p. followed by four consecutive i.p. injections of 10 μ g of TNF without adjuvant. Eight weeks after the last injection, the mouse was boosted i.p. with 10 μ g of TNF.

Four days later, the mouse was sacrificed, the spleen was obtained and a spleen cell suspension was prepared. Spleen cells were fused with cells of the nonsecreting hybridoma, Sp2/0 (ATCC CRL1581), at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. After incubation at 37°C for 6 hours, the fused cells were distributed in 0.2 ml aliquots into 96-well plates at concentrations of 2×10^4 SP2/0 cells per well. Feeder cells, in

the form of 5×10^4 normal BALB/c spleen cells, were added to each well.

The growth medium used consisted of RPMI-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin (GIBCO Laboratories) and, for selection, hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim).

A solid-phase radioimmunoassay (RIA) was employed for screening supernatants for the presence of mAbs specific for rhTNF α . This assay is described in Example II, below. The background binding in this assay was about 500 cpm. A supernatant was considered positive if it yielded binding of 2000 cpm or higher.

Of 322 supernatants screened, 25 were positive by RIA. Of these 25, the one with the highest binding (4800 cpm) was designated A2. Positive wells were subcloned at limiting dilution on mouse feeder cells. Upon further analysis of the supernatants in neutralization assays, A2 was found to be the only positive clone showing potent inhibiting and/or neutralizing activity. Thus, the hybridoma line A2 was selected. This line was maintained in RPMI-1640 medium with 10% FBS (GIBCO), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin.

Alternatively, anti-TNF antibodies which inhibit TNF biological activity can be screened by binding to peptide including at least 5 amino acids of residues 87-108 or both residues 59-80 and 87-108 of TNF (of SEQ ID NO:1) or combinations of peptides contained therein, which are used in place of the rTNF protein, as described above.

EXAMPLE II

Characterization of an Anti-TNF antibody of the present invention.

A. Radioimmunoassays

E. coli-derived rhTNF was diluted to 1 μ g/ml in BCB buffer, pH 9.6, and 0.1 ml of the solution was added to each

assay well. After incubation at 4°C overnight, the wells were washed briefly with BCB, then sealed with 1% bovine serum albumin (BSA) in BCB at 37°C for 1 hr. The wells were then washed 3 times with PBS containing 0.05% Tween-20 (PBS-Tween), and 70 µl diluted A2 ascites fluid was added to each well. The wells were incubated for 2 hr at 37°C, and washed 3 times with PBS-Tween. Thereafter, approximately 50,000 cpm of ¹²⁵I-labeled F(ab')₂ fragment of sheep anti-mouse Ig antibodies in 50 µl of PBS-Tween containing 1% BSA was added to each well, and the wells were incubated for an additional 2 hr at 37°C. The wells were washed 4 times with PBS-Tween, cut out and counted individually. Results of two determinations are shown in Figure 1.

rhTNF at 5 µg/ml in PBS was heated to 60°C. At various time points, aliquots of the heat-treated TNF preparation were quickly cooled to 4°C, diluted 5-fold in BCB, and used to coat the RIA microplate wells. The RIA was carried out exactly as described above. Results from two determinations are shown in Figure 2. As incubation at 60°C substantially reduced the biological activity of hTNFα, this experiment shows mAb A2 fails to bind heat-inactivated human TNFα.

B. Neutralization Assays

Samples of A2 and cA2 were purified by protein A affinity chromatography from hybridoma tissue culture supernatants of cell lines designated C134A and C168A (described above), respectively, and diafiltered in phosphate buffered saline pH 7.2 (PBS).

The toxic effect of TNF on certain tumor cell lines has been adapted as an in vitro measure of TNF levels in laboratory samples and biological fluids. The assay method of Feinman *et al.*, *J Immunol* **138**:635-640 (1987), as modified by Aderka *et al.*, *J. Immunol.* **143**:3517-3523 (1989), employing the TNF-sensitive target cell A673 (a human rhabdomyosarcoma cell line), was used to investigate the ability of A2 to inhibit or neutralize TNF toxicity.

Cultured human A673/6 cells were incubated with 40 pg/ml of natural (Genzyme, Boston, MA) or recombinant (Suntory, Osaka, Japan) human TNFα with varying concentrations of mAb A2 in the presence of 20 µg/ml cycloheximide at 39°C overnight.

Controls included medium alone or medium + TNF in each well. Cell death was measured by staining with naphthol blue-black, and the results read spectrophotometrically at 630 nm. Absorbance at this wave length correlates with the number of
5 live cells present.

It was found that A2 inhibited or neutralized the cytotoxic effect of both natural and rhTNF in a dose-dependent manner (Figure 3).

In another experiment, the specificity of this
10 inhibiting and/or neutralizing activity was tested. A673/6 cells were seeded at 3×10^4 cells/well 20 hr before the TNF bioassay. Two-fold serial dilutions of rhTNF, E. coli-derived recombinant human lymphotoxin (TNF β), and E. coli-derived recombinant murine TNF were prepared. The A2 hybridoma
15 supernatant was added to an equal volume of the diluted TNF preparations, and the mixtures were incubated at room temperature for 30 min. Aliquots of 0.1 ml were transferred to the wells containing A673/6 cells, 20 μ g/ml of cycloheximide was added, and the cells were incubated at 39°C overnight. The
20 cells were then fixed and stained for evaluation of cytotoxicity. The results indicate that mAb A2 specifically inhibited or neutralized the cytotoxicity of rhTNF α , whereas it had no effect on human lymphotoxin (TNF β) (Figure 4) or murine TNF (Figure 5).

25 Experiments were next performed to analyze the cross-reactivity of mAb A2 with TNF derived from non-human primates.

Monocytes isolated from B514 (baboon), J91 (cynomolgus) and RH383 (rhesus) blood by Ficoll gradient centrifugation and
30 adherence, were incubated at 1×10^5 cells/well in RPMI 1640 medium with 5% FBS and 2 μ g/ml of E. coli LPS for 3 or 16 hr at 37°C to induce TNF production. Supernatants from duplicate wells were pooled and stored at 4°C for less than 20 hr until the TNF bioassay was performed, as described above, using A673/6
35 cells. Two-fold dilutions of the culture supernatants were mixed with either medium or purified mAb A2 at a final concentration of 1 μ g/ml, incubated at room temperature for 30 min and aliquots transferred to the indicator cells. The results showed that mAb A2 failed to significantly inhibit or neutralize the

cytotoxic activity of TNF produced by baboon, cynomolgus and rhesus monkey monocytes.

A further experiment was conducted with chimpanzee TNF. Monocytes isolated from CH563 (chimpanzee) blood were incubated as described above to generate TNF-containing supernatants. The ability of 10 μ g/ml of mAb A2 to inhibit or neutralize the bioactivity of these supernatants was assayed as above. Human TNF was used as a positive control. Results, shown in Figure 6, indicate that mAb A2 had potent inhibiting and/or neutralizing activity for chimpanzee TNF, similar to that for human TNF (Figure 7).

The inhibiting and/or neutralizing activity of mAb A2 was compared with three other murine mAbs specific for human TNF, termed TNF-1, TNF-2 and TNF-3, and a control mAb. Two-fold serial dilutions of purified mAbs were mixed with rhTNF (40 pg/ml), incubated at room temperature for 30 min, and aliquots tested for TNF bioactivity as above. It was found that mAbs TNF-1, TNF-2 and TNF-3 each had a similar moderate degree of inhibiting and/or neutralizing activity. In contrast, mAb A2 had much more potent inhibiting and/or neutralizing activity.

EXAMPLE III

General Strategy for Cloning Antibody V and C Genes

The strategy for cloning the V regions for the H and L chain genes from the hybridoma A2, which secretes the anti-TNF antibody described above, was based upon the linkage in the genome between the V region and the corresponding J (joining) region for functionally rearranged (and expressed) Ig genes. J region DNA probes can be used to screen genomic libraries to isolate DNA linked to the J regions. Although DNA in the germline configuration (i.e., unrearranged) would also hybridize to J probes, this DNA would not be linked to a Ig V region sequence and can be identified by restriction enzyme analysis of the isolated clones.

The cloning utilized herein was to isolate V regions from rearranged H and L chain genes using J_H and J_L probes. These clones were tested to see if their sequences were expressed in the A2 hybridoma by Northern analysis. Those clones that contained expressed sequence were cloned into

expression vectors containing human C regions and transfected into mouse myeloma cells to determine if an antibody was produced. The antibody from producing cells was then tested for binding specificity and functionally compared to the A2 murine antibody.

EXAMPLE IV

Construction of a L Chain Genomic Library

To isolate the L chain V region gene from the A2 hybridoma, a size-selected genomic library was constructed using the phage lambda vector charon 27. High molecular weight DNA was isolated from A2 hybridoma cells and digested to completion with restriction endonuclease HindIII. The DNA was then fractionated on a 0.8% agarose gel and the DNA fragments of three different size ranges of approximately 3 kb, 4 kb and 6 kb were isolated from the gel by electroelution. The size ranges for library construction were chosen based upon the size of HindIII fragments that hybridized on a southern blot with the J_k probe. After phenol/chloroform extraction and ethanol precipitation, the DNA fragments from each size class were ligated with lambda charon 27 arms and packaged into phage particles in vitro using Gigapack Gold from Stratagene (LaJolla, CA).

These libraries were screened directly at a density of approximately 20,000 plaques per 150 mm petri dish using a ^{32}P -labeled J_k probe. The mouse L chain J_k probe was a 2.7 kb HindIII fragment containing all five J_k segments. The probe was labeled with ^{32}P by random priming using a kit obtained from Boehringer Mannheim. Free nucleotides were removed by centrifugation through a Sephadex G-50 column. The specific activities of the probe was approximately 10^9 cpm/ μg .

Plaque hybridizations were carried out in 5x SSC, 50% formamide, 2x Denhardt's reagent, and 200 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA at 42°C for 18-20 hours. Final washes were in 0.5x SSC, 0.1% SDS at 65°C. Positive clones were identified after autoradiography.

EXAMPLE V

Construction of H Chain Genomic Library

To isolate the V region gene for the A2 H chain, a genomic library was constructed in the lambda gt10 vector system. High molecular weight DNA was digested to completion with restriction endonuclease EcoRI and fragments of approximately 7.5 kb were isolated after agarose gel electrophoresis. These fragments were ligated with lambda gt10 arms and packaged into phage particles in vitro using Gigapack Gold.

This library was screened at a density of 20,000 plaques per 150 mm plate using a J_H probe. The J_H probe was a 2kb BamHI/EcoRI fragment containing both J3 and J4 segments. The probe was labeled as in Example III and had a similar specific radioactivity. Hybridization and wash conditions were identical to those used in Example III.

EXAMPLE VI

Cloning of the TNF-Specific V gene regions

Several positive clones were isolated from the H and L chain libraries after screening approximately 10⁶ plaques from each library using the J_H and J_k probes, respectively. Following plaque purification, bacteriophage DNA was isolated for each positive clone, digested with either EcoRI (H chain clones) or HindIII (L chain clones) and fractionated on 1% agarose gels. The DNA was transferred to nitrocellulose and the blots were hybridized with the J_H or the J_k probe.

Several H chain clones were obtained that contained 7.5 kb EcoRI DNA fragments that hybridized to the J_H probe. For the light chain libraries, several clones from each of the three size-selected libraries were isolated that contained HindIII fragments that hybridize to the J_k probe. For the L chain, several independently derived HindIII fragments of 2.9 kb from the 2 kb library hybridized with a 1250 bp mRNA from A2, but not with SP2/0 mRNA (see Example VII). In addition, several HindIII fragments derived from the 4 kb library hybridized both to the A2 mRNA and the fusion partner mRNA. A 5.7 kb HindIII fragment from the 6 kb library did not hybridize to either RNA.

The observed lengths of hybridizing A2 mRNA were the correct sizes for H and L chain mRNA, respectively. Because the RNA expression was restricted to the A2 hybridoma, it was

assumed that the 7.5 kb H chain fragments and the 2.9 kb L chain fragments contained the correct V region sequences from A2. One example of each type was chosen for further study. The important functional test is the demonstration that these V regions sequences, when combined with appropriate C region sequences, are capable of directing the synthesis of an antibody with a specificity and affinity similar to that of the murine A2 antibody.

The 7.5 kb H chain fragment and the 2.9 kb L chain fragment were subcloned into plasmid vectors that allow expression of the chimeric mouse/human proteins in murine myeloma cells (see Examples VIII and IX). These plasmids were co-transfected into SP2/0 cells to ascertain if intact antibody molecules were secreted, and if so, if they were of the correct specificity and affinity. Control transfections were also performed pairing the putative anti-TNF H chain with an irrelevant, but expressed, L chain; the putative anti-TNF L chain was also paired with an irrelevant, but expressed, H chain. The results indicated that the 7.5 kb H chain fragment could be expressed, whereas the 2.9 kb L chain fragment could not. This was confirmed by DNA sequence analysis that suggested portions of the coding region were not in the proper amino acid reading frame when compared to other known L chain amino acid sequences.

Because the 2.9 kb HindIII fragment appeared not to contain a functional V gene, the 4.0 kb and 5.7 kb HindIII fragments isolated from L chain libraries were cloned into expression vectors and tested for expression of chimeric antibody after co-transfection with the 7.5 kb H chain. The 5.7 kb HindIII fragment was incapable of supporting antibody expression, whereas the 4.0 kb HindIII fragment did support antibody expression. The antibody resulting from the co-transfection of the 7.5 kb putative H chain V region and the 4.0 kb L chain V region was purified, tested in solid phase TNF binding assay, and found to be inactive. It was concluded that the V region contained on the 4.0 kb HindIII fragment was not the correct anti-TNF V regions, but was contributed to the hybridoma by the fusion partner. This was subsequently

confirmed by sequence analysis of cDNA derived from the A2 hybridoma and from the fusion partner.

Other independently derived L chain clones containing 2.9 kb HindIII fragments that hybridized with A2 mRNA were characterized in more detail. Although the restriction maps were similar, the clones fell into two classes with respect to the presence or absence of an AccI enzyme site. The original (non-functional) 2.9 kb fragment (designated clone 8.3) was missing an AccI site present in some other clones (represented by clone 4.3). The DNA sequence of clone 4.3 was extremely similar to clone 8.3, but contained a single amino acid reading frame with close homology to known L chains, unlike clone 8.3. The 2.9 kb HindIII fragment from clone 4.3 was subcloned into the L chain expression vector and co-transfected with the putative anti-TNF H chain into SP2/0 cells. An antibody was synthesized, purified and tested in the solid phase TNF binding assay. This antibody bound to TNF, and therefore, the clone 4.3 L chain V region was assumed to be the correct one.

The A2 murine hybridoma has been shown to contain at least four rearranged L chain V region genes. At least two of these are expressed as proteins: clone 4.3 (the correct anti-TNF L chain gene) and the gene contained in the 4.0 kb HindIII fragment (contributed by the fusion partner). The expression of two L chains implies that the resulting antibody secreted from the murine hybridoma is actually a mixture of antibodies, some using the correct L chain, some using the incorrect L chain, and some using one of each. The presence of two different L chains in the murine A2 antibody has been confirmed by SDS gel and N-terminal protein sequence analysis of the purified antibody. Because construction of the chimeric A2 antibody involves cloning the individual H and L chain genes and expressing them in a non-producing cell line, the resulting antibody will have only the correct L chain and therefore should be a more potent antibody (see Examples X, XI and XII).

EXAMPLE VII

Northern Analysis of Cloned DNA

Cloned DNA corresponding to the authentic H and L chain V regions from the A2 hybridoma would be expected to

hybridize to A2 mRNA. Non-functional DNA rearrangements at either the H or L chain genetic loci should not be expressed.

Ten μ g total cellular RNA was subjected to electrophoresis on 1% agarose/formaldehyde gels (Sambrook et al, supra) and transferred to nitrocellulose. Blots were hybridized with random primed DNA probes in 50% formamide, 2x Denhardt's solution, 5x SSC, and 200 μ g/ml denatured salmon sperm DNA at 42°C for 10 hours. Final wash conditions were 0.5 x SSC, 0.1% SDS at 65°C.

The subcloned DNA fragments were labeled with 32 P by random priming and hybridized to Northern blots containing total RNA derived from A2 cells or from cells of SP2/0, the fusion partner parent of A2. The 7.5 kb EcoRI H chain fragment hybridized with a 2 kb mRNA from A2, but not with SP2/0 mRNA. Similarly, the 2.9 kb L chain HindIII fragment (clone 4.3) hybridized with a 1250 bp mRNA from A2, but not with SP2/0 mRNA. The observed lengths of A2 mRNA hybridizing were the correct sizes for H and L chain mRNA, respectively, confirming that the V region sequences on these DNA fragments are expressed in A2 hybridoma cells.

EXAMPLE VIII:

Construction of Expression Vectors

The putative L (clone 4.3) and H chain V genes described above were joined to human kappa and gamma1 constant region genes in expression vectors. The 7.5 kb EcoRI fragment corresponding to the putative V_H region gene from A2 was cloned into an expression vector containing the human C_{gamma1} gene and the Ecogpt gene to yield the plasmid designated pA2HG1apgpt (see Figure 8).

The 2.9 kb putative V_L fragment from clone 4.3 was cloned into a vector containing the human kappa C_k gene and the Ecogpt gene to allow selection in mammalian cells. The resulting plasmid was designated pA2HuKapgpt (See Figure 8).

EXAMPLE IX

Expression of Chimeric Antibody Genes

To express the chimeric H and L chain genes, the expression plasmids were transfected into cells of the non-producing mouse myeloma cell line, SP2/0. Plasmid DNA to be transfected was purified by centrifuging to equilibrium in ethidium bromide/cesium chloride gradients twice. Plasmid DNA (10-50 μ g) was added to 10^7 SP2/0 cells in medium containing Hank's salts, and the mixture was placed in a BioRad electroporation apparatus. Electroporation was performed at 20 volts, following which the cells were plated in 96 well microtiter plates.

Mycophenolic acid selection was applied after 24 hours and drug resistant colonies were identified after 1-2 weeks. Resistant colonies were expanded to stable cell lines and tissue culture supernatant from these cell lines was tested for antibody using an ELISA assay with goat anti-human IgG Fc antibody and goat anti-human H+L conjugated with alkaline phosphatase (obtained from Jackson Laboratories).

The chimeric A2 antibody was purified from tissue culture supernatant by Protein A- Sepharose chromatography. The supernatant was adjusted to 0.1M Tris, 0.002M EDTA, pH 8.0 and loaded on a Protein A-Sepharose column equilibrated in the same buffer. The IgG was eluted with 0.1M citrate, pH 3.5, inhibited or neutralized with 1M Tris, and dialyzed into phosphate buffered saline (PBS).

The purified chimeric antibody was evaluated for its binding and inhibiting and/or neutralizing activity.

EXAMPLE X

Specificity of an Anti-TNF Chimeric Antibody

Since the antigen binding domain of cA2 was derived from murine A2, these mAbs would be expected to compete for the same binding site on TNF. Fixed concentrations of chimeric A2 and murine mAb A2 were incubated with increasing concentrations of murine and chimeric A2 competitor, respectively, in a 96-well microtiter plate coated with rhTNF (Dainippon, Osaka, Japan). Alkaline-phosphatase conjugated anti-human immunoglobulin and anti-mouse immunoglobulin second antibodies were used to detect

the level of binding of chimeric and murine A2, respectively. Cross-competition for TNF antigen was observed in this solid-phase ELISA format (Figure 9). This finding is consistent with the expected identical epitope specificity of cA2 and murine A2.

The affinity constant for binding of mouse mAb A2 and cA2 to rhTNF α was determined by Scatchard analysis (see, for example, Scatchard, G., Ann. N.Y. Acad. Sci. 51:660 (1949)). The results are shown in Figure 10. This analysis involved measuring the direct binding of ¹²⁵I labelled cA2 to immobilized rhTNF α in a 96-well plate. The antibodies were each labelled to a specific activity of about 9.7 μ Ci/ μ g by the iodogen method. An affinity constant (K_a) of 0.5×10^9 liters/mole was calculated for the mouse mAb A2. Unexpectedly, the chimeric A2 antibody had a higher affinity, with a K_a of 1.8×10^9 liters/mole. Thus, the chimeric anti-TNF α antibody of the present invention was shown to exhibit a significantly higher affinity of binding to human TNF α than did the parental murine A2 mAb. This finding was surprising, since murine and chimeric antibodies, fragments and regions would be expected to have affinities that are equal to or less than that of the parent mAb.

Such high affinity anti-TNF antibodies, having affinities of binding to TNF α of at least 1×10^8 M⁻¹, more preferably at least 1×10^9 M⁻¹ (expressed as K_a) are preferred for immunoassays which detect very low levels of TNF in biological fluids. In addition, anti-TNF antibodies having such high affinities are preferred for therapy of TNF- α -mediated conditions or pathology states.

The specificity of cA2 for TNF was confirmed by testing for cross-neutralization of human lymphotoxin (TNF- β). Lymphotoxin shares some sequence homology and certain biological activities, for example, tumor cell cytotoxicity, with TNF (Pennica, D. et al., Nature 312:724-729 (1984)). Cultured human A673 cells were incubated with increasing concentrations of human lymphotoxin (Genentech, San Francisco, CA) with or without 4 μ g/ml chimeric A2 in the presence of 20 μ g/ml cycloheximide at 39°C overnight. Cell death was measured by vital staining with naphthol blue-black, as above. The results indicated that cA2 was ineffective at inhibiting and/or neutralizing human

lymphotoxin, confirming the TNF α -specificity of the chimeric antibody.

The ability of A2 or cA2 to react with TNF from different animal species was also evaluated. As mentioned earlier, there are multiple epitopes on human TNF to which inhibiting and/or neutralizing mAbs will bind (Moller, A. et al., supra). Human TNF has bioactivity in a wide range of host animal species. However, certain inhibiting and/or neutralizing epitopes on human TNF are conserved amongst different animal species and others appear to be restricted to humans and chimpanzees.

Neutralization experiments utilized endotoxin-activated cell supernatants from freshly isolated human, chimpanzee, rhesus and cynomolgus monkey, baboon, pig, dog, rabbit, or rat monocytes as the TNF source. As discussed above, murine mAb A2 inhibited or neutralized activity of only human and chimpanzee TNF, and had no effect on TNF derived from other primates and lower animals. A2 also did not inhibit or neutralize the cytotoxic effect of recombinant mouse TNF.

Thus, the epitope recognized by A2 is one shared by human and chimpanzee TNF α . Chimeric A2 was also tested in this manner for cross-reactivity with monocyte-derived TNF from rat, rabbit, dog and pig, as well as with purified recombinant mouse TNF α , and natural and recombinant human TNF α . Chimeric A2 only inhibited or neutralized natural and recombinant human TNF α . Therefore, cA2 appears to share species specificity with murine A2.

EXAMPLE XI

In Vitro Activity and Neutralization Efficacy of a Chimeric Anti-TNF Antibody

Both the murine and chimeric anti-TNF α antibodies, A2 and cA2 were determined to have potent TNF-inhibiting and/or neutralizing activity. In the TNF cytotoxicity assay described above, murine A2, at a concentration of about 125 ng/ml completely inhibited or neutralized the biological activity of a 40 pg/ml challenge of rhTNF α . Two separate determinations of inhibiting and/or neutralizing potency, expressed as the 50% Inhibitory Dose (ID50) were determined to be 15.9 \pm 1.01 and

17.9 \pm 1.6 ng/ml (Mean \pm Std error). Thus the mAb A2 has an ID50 of about 17 ng/ml.

In this same experimental system, three other murine anti-TNF α antibodies (termed TNF-1, TNF-2 and TNF-3) of comparable binding affinity to TNF were found to have ID50 values of 1-2 orders of magnitude greater, and thus were significantly less potent in neutralization than A2.

The ability of cA2 to inhibit or neutralize human TNF α bioactivity in vitro was tested using the bioassay system described above. Cultured A673 cells were incubated with 40 pg/ml natural (Genzyme, Boston, MA) or recombinant (Suntory, Osaka, Japan) human TNF with or without antibody overnight as above, and cell death was measured by vital staining. As expected based upon the above results with the A2 mouse mAb, cA2 also inhibited or neutralized both natural and rhTNF in a dose-dependent manner in the cytotoxicity assay (Figure 11). In this assay format, levels of cA2 as low as 125 ng/ml completely abolished the toxic activity of TNF. Upon repeated analysis, the cA2 exhibited greater TNF-inhibiting and/or neutralizing activity than did the parent murine A2 mAb. Such inhibiting and/or neutralizing potency, at antibody levels below 1 μ g/ml, can easily be attained in the blood of a subject to whom the antibody is administered. Accordingly, such highly potent inhibiting and/or neutralizing anti-TNF antibodies, in particular the chimeric antibody, are preferred for therapeutic use in TNF α -mediated pathologies or conditions.

As mentioned above, TNF induces cellular secretion of IL-6. Furthermore, there is evidence that IL-6 is involved in the pathophysiology of sepsis, although the precise role of IL-6 in that syndrome is unclear (Fong, Y. et al., J Exp Med 170:1627-1633 (1989); Starnes Jr., H.F. et al., J Immunol 145:4185-4191 (1990)). The ability of cA2 to inhibit or neutralize TNF-induced IL-6 secretion was evaluated using cultured human diploid FS-4 fibroblasts. The results in Table 1 show that cA2 was effective in blocking IL-6 secretion in cells that had been incubated overnight with TNF. TNF-induced IL-6 secretion was not inhibited in the absence of a mAb or in the presence of a control mAb specific for an irrelevant antigen.

TABLE 1

IN VITRO NEUTRALIZATION OF TNF-INDUCED IL-6 SECRETION

Antibody	TNF Concentration (ng/ml)			
	0	0.3	1.5	7.5
None	<0.20	1.36	2.00	2.56
Control mAb	<0.20	1.60	1.96	2.16
cA2	<0.20	<0.20	<0.20	0.30

Values represent mean concentrations of IL-6 of duplicate wells, in ng/ml. RhTNF (Suntory, Osaka, Japan), with or without 4 μ g/ml antibody, was added to cultures of FS-4 fibroblasts and after 18 h, the supernatant was assayed for IL-6 using the Quantikine Human IL-6 Immunoassay (from R&D Systems, Minneapolis, MN). Control mAb = chimeric mouse/human IgG1 anti-platelet mAb (7E3).

The ability of TNF to activate procoagulant and adhesion molecule activities of endothelial cells (EC) is thought to be an important component of pathology pathophysiology. In particular, this may be associated with the vascular damage, disseminated intravascular coagulation, and severe hypotension that is associated with the sepsis syndrome. Therefore, the ability of cA2 to block TNF-induced activation of cultured human umbilical vein endothelial cells (HUVEC) was evaluated. TNF stimulation of procoagulant activity was determined by exposing intact cultured HUVEC cells to TNF (with or without antibody) for 4 hours and analyzing a cell lysate in a human plasma clotting assay. The results in Table 2 show the expected upregulation by TNF of HUVEC procoagulant activity (reflected by a decreased clotting time). Chimeric antibody cA2 effectively inhibited or neutralized this TNF activity in a dose-dependent manner.

TABLE 2

IN VITRO NEUTRALIZATION OF TNF-INDUCED
PROCOAGULANT ACTIVITY

Antibody	$\mu\text{g/ml}$	TNF Concentration (ng/ml)		
		250	25	0
None	-	64 \pm 4*	63 \pm 1	133 \pm 13
Control Ab	10.00	74 \pm 6	N.D.	178 \pm 55
cA2	10.00	114 \pm 5	185 \pm 61	141 \pm 18
cA2	3.30	113 \pm 2	147 \pm 3	N.D.
cA2	1.10	106 \pm 1	145 \pm 8	N.D.
A2	0.37	73 \pm 17	153 \pm 4	N.D.
cA2	0.12	64 \pm 1	118 \pm 13	N.D.

* Values represent mean plasma clotting time, in seconds (\pm S.D.). Clotting time was determined in normal human plasma after addition of the rhTNF (Dainippon, Osaka, Japan) with or without antibody-treated HUVEC lysate and Ca^{++} at 37°C. N.D. = Not done. Control Ab is a chimeric mouse/human IgG1 anti-CD4 antibody.

In addition to stimulating procoagulant activity, TNF also induces surface expression of endothelial cell adhesion molecules such as ELAM-1 and ICAM-1. The ability of cA2 to inhibit or neutralize this activity of TNF was measured using an ELAM-1 specific detection radioimmunoassay. Cultured HUVEC were stimulated with 250 ng/ml rhTNF (Dainippon, Osaka, Japan) with or without antibody at 37°C overnight in a 96-well plate format. Surface expression of ELAM-1 was determined by sequential addition of a mouse anti-human ELAM-1 mAb and ^{125}I -labelled rabbit anti-mouse immunoglobulin second antibody directly to culture plates at 4°C.

As shown in Figure 12, TNF induced the expression of ELAM-1 on the surface of cultured HUVEC cells, and this activity was again effectively blocked in a dose-related manner by cA2.

Finally, TNF is known to stimulate mitogenic activity in cultured fibroblasts. Chimeric A2 inhibited or neutralized

TNF-induced mitogenesis of human diploid FS-4 fibroblasts cultures, confirming the potent inhibiting and/or neutralizing capability of cA2 against a broad spectrum of in vitro TNF biological activities.

EXAMPLE XII

In Vivo Activity and Efficacy of cA2 Antibody

The highly restrictive species cross-reactivity of cA2 severely limits the ability to test the in vivo efficacy of this antibody in animals other than humans or chimpanzees.

Nevertheless, evidence that the potent in vitro inhibiting and/or neutralizing capability of cA2 is manifest in vivo was desirable. Earlier animal studies showed that administration of TNF to experimental animals mimics the pathology state obtained with either Gram-negative bacterial infection or direct endotoxin administration (Tracey, K.J. et al., 1986. supra; Tracey, K.J. et al., 1987, supra; Lehmann, V. et al., supra).

An in vivo model wherein lethal doses of human TNF are administered to galactosamine-sensitized mice (Lehmann, V. et al., supra) was adapted for testing the capability of cA2 to inhibit or neutralize TNF in vivo. An i.p. challenge with 5 μ g (0.25 mg/kg) of rhTNF resulted in 80-90 percent mortality in untreated control animals and in animals treated i.v. 15-30 minutes later with either saline or 2 mg/kg control antibody (a chimeric IgG1 derived from murine 7E3 anti-platelet mAb). In contrast, treatment with cA2 reduced mortality to 0-30 percent with 0.4 mg/kg of antibody, and to 0-10 percent with 20 mg/kgs. These results, summarized in Table 3, indicate that cA2 was capable of inhibiting and/or neutralizing the biological activity of TNF in vivo as well as in vitro.

TABLE 3

PREVENTION OF HUMAN TNF-INDUCED LETHALITY BY CHIMERIC A2

Outcome (Survivors/Total)

5	<u>Antibody</u>	<u>Experiment #1</u>	<u>Experiment #2</u>
	None	1/10	N.D.
	Control Ab, 2 mg/kg	2/10	1/10
	cA2 (2 mg/kg)	9/10 (p=0.0055)	10/10 (p=0.0001)
10	cA2 (0.4 mg/kg)	7/10 (p=0.07)	10/10 (p=0.0001)
15	Female C3H/HeN mice were administered 5 µg rhTNF (Dainippon, Osaka, Japan) + 18 mg galactosamine i.p. and antibody was administered 15-30 minutes later i.v. Deaths were recorded 48 h post-challenge. Control MAb = chimeric mouse/human IgG1 anti-platelet MAb (7E3). N.D. = not done. p values refer to comparison with the control Ab.		

EXAMPLE XIII:

Determination of amino acid sequences (epitope) on
human TNF-α recognized by cA2 mAb

20 Reagents The following reagents are readily available from commercial sources. FMOC-L-Ala-OPfp, FMOC-L-Cys(Trt)-OPfp, FMOC-L-Asp(OtBu)-OPfp, FMOC-L-Glu(OtBu)-OPfp, FMOC-L-Phe-OPfp, FMOC-Gly-OPfp, FMOC-L-His(Boc)-OPfp, FMOC-L-Ile-OPfp, FMOC-L-Lys(Boc)-OPfp, FMOC-L-Leu-OPfp, FMOC-L-Asn-OPfp,

25 F M O C - L - P r o - O P f p , F M O C - L - G l n - O P f p ,
FMOC-L-Arg(Mtr)-OPfp, F M O C - L - S e r (t B u) - O D h b t ,
FMOC-L-Thr(tBu)-ODhbt, FMOC-L-Val-OPfp, FMOC-L-Trp-OPfp,
FMOC-L-Try(tBu)-OPfp, and 1-hydroxybenotriazol (HOBT) were obtained from Cambridge Research Biochemicals. Piperidine and was

30 obtained from Applied Biosystems, Inc. 1-Methyl-2-Pyrrolidinone (NMP) was obtained from EM Science; Methanol from JT Baker; Acetic Anhydride from Applied Biosystems, Inc., Trifluoroacetic acid (TFA) from Applied Biosystems, Inc.; Diisopropylamine (DIEA), Triethylamine, Dithiothreitol (DTT) and Anisole from Aldrich and

35 Hydrochloric Acid (HCl) from JT Baker.

Abbreviations: FMOC, 9-fluorenylmethoxycarbonyl; tBu t-butyl ether; OtB, t-butyl ester; Boc, t-butyloxycarbonyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; Trt, trityl; OPfp, pentafluorophenylester; ODhbt. oxo-benzotriazone ster;

A chimeric antibody of the present invention, designated cA2, was used to determine which portions of the TNF amino acid sequence were involved in inhibitory binding by the antibody by epitope mapping, whereby the amino acid sequences of TNF- α recognized by cA2 have been identified.

The complete primary sequence of human TNF α , according to Pennica et al, Nature 312:724-729 (1984) is shown in Figure 13. Overlapping decapeptides beginning with every second amino acid and covering the entire amino acid sequence of human TNF- α were synthesized on polyethylene pins using the method of Gysen (Gysen et al., Peptides: Chemistry and Biological, Proceedings of the Twelfth American Peptide Symposium, p. 519-523, Ed, G.R. Marshall, Escom, Leiden, 1988). Sets of peptide pins bearing free N-terminal amino groups and acetylated N-terminal amino groups were individually prepared. Both sets of peptide pins were incubated in solutions containing the anti-TNF mAb cA2 to determine the amino acid sequences that make up the cA2 epitope on human TNF- α , as described below. Figure 14A shows the results of binding to the overlapping decapeptides that comprise the entire sequence of human TNF α . The O.D. (optical density) correlates directly with the increased degree of cA2 binding. Figure 14B shows the results of binding of cA2 to the same set of peptide pins in the presence of human TNF α . This competitive binding study delineates peptides which may show non-specific binding to cA2.

There are at least two non-contiguous peptide sequences of TNF- α recognized by cA2. Using the conventional protein numbering system wherein the N-terminal amino acid is number 1, the cA2 mAb recognizes an epitope composed at least in part of amino acids located within residues 87-108 or both residues 59-80 and 87-108 of TNF (of SEQ ID NO:1). Figure 15 presents these non-contiguous sequences within the TNF sequence. These sequences are also shown in a space filling model in Figure 16B, along with a space filling model of the TNF monomer shown in Figure 16A.

Unexpectedly, the mAb cA2 blocks the action of TNF- α without binding to the putative receptor binding locus, which can include one or more of, e.g., 11-13, 37-42, 49-57 or 155-157 of hTNF α (of SEQ ID NO:1). Preferred anti-TNF mAbs are those that inhibit this binding of human TNF- α to its receptors by virtue of their ability to bind to one or more of these peptide sequences.

These antibodies can block the activity of TNF by virtue of binding to the cA2 epitope, such binding demonstrated to inhibit TNF activity. The identification of those peptide sequences recognized by cA2 provides the information necessary to generate additional monoclonal antibodies with binding characteristics and therapeutic utility that parallel the embodiments of this application.

Peptide Pin Synthesis Using an epitope mapping kit purchased from Cambridge Research Biochemicals, Inc. (CRB), dodecapeptides corresponding to the entire sequence of human TNF- α were synthesized on polyethylene pins.

A synthesis schedule was generated using the CRB epitope mapping software. Prior to the first amino acid coupling, the pins were deprotected with a 20% piperidine in NMP solution for 30 minutes at room temperature. After deprotected, the pins were washed with NMP for five minutes at room temperature, followed by three methanol washes. Following the wash steps, the pins were allowed to air dry for at least 10 minutes.

The following procedure was performed for each coupling cycle:

- 1) The amino acid derivatives and the HOBt were weighed out according to the weights required in the synthesis schedule.

- 2) The HOBt was dissolved in the appropriate amount of NMP according to the synthesis schedule.

- 3) The amino acid derivatives were dissolved in the recommended amount of HOBt solution and 150 microliters were pipetted into the appropriate wells as directed by the well position sheet of the synthesis schedule.

- 4) The blocks containing the pins were placed into the wells, and the "sandwich" units stored in plastic bags in a 30°C water bath for 18 hours.

- 5) The pins were removed from the wells and washed once (for 5 minutes) with NMP, three times (for two minutes) with methanol and air dried for 10 minutes.

- 6) The pins were deprotected as described above and the procedure repeated.

To acetylate the peptides on one block of pins, the peptide pins were washed, deprotected and treated with 150 microliters of a solution containing NMP; acetic

anhydride:triethylamine (5:2:1) for 90 minutes at 30°C, followed by the washing procedure outlined above. The second set of peptide pins was deprotected by not acetylated to give free N-terminal amino groups.

5 The final deprotection of the peptides to remove the side chain protecting groups was done using a mixture of TFA:anisole:dithiothreitol, 95:2.5:2.5 (v/v/w) for four hours at ambient temperature. After deprotection, the pins were air dried for 10 minutes, followed by a 15 minute sonication in a solution of 0.1% HCl in methanol/distilled water (1:1). The pins dried over night and were then ready for testing.

ELISA Assay for cA2 Binding to TNF- α Peptide PINs

15 Reagents: Disruption Buffer: Sodium dihydrogen phosphate (31.2 g, Sigma cat # S-0751 or equivalent) and sodium dodecylsulfate (20.0 g, Sigma cat # L-3771 or equivalent) were dissolved in 2.0 L of milliQ water. The pH was adjusted to 7.2 \pm 0.1 with 50% w/w sodium hydroxide (VWR cat # VW6730-3 or equivalent).

20 Blocking Buffer: Sodium dihydrogen phosphate (0.39 g, Sigma cat #S-0751 or equivalent) disodium hydrogen phosphate (1.07 g, Baker cat # 3828-1 or equivalent) and sodium chloride (8.50 g, Baker cat # 3624-5 or equivalent) were dissolved in 1.0 L of milliQ water. The pH was adjusted to 7.2 \pm 0.1 with 50% w/w sodium hydroxide (VWR cat VW6730-3 or equivalent). Chicken egg albumin (10.0 g, Sigma cat #A-5503 or equivalent) and bovine serum albumin (10.0 g, Sigma, cat #A-3294 or equivalent) were dissolved at room temperature with gentle stirring. The solution was filtered, and to the solution was added Tween 20 (2.0 ml, Sigma cat #P-13.79 or equivalent). The solution was stirred gently at room temperature for 30 min, filtered and stored at 40°.

30 PBS/Tween 20: A 10 x concentrate was prepared by dissolving sodium dihydrogen phosphate (3.90 g, Sigma cat # S-0751 or equivalent), disodium hydrogen phosphate (10.70 g, Baker cat #3828-1 or equivalent) and sodium chloride (85.0 g, Baker cat #3624-5 or equivalent) in 1.0 L of milliQ water. The pH was adjusted to 7.2 \pm 0.1 with 50% w/w sodium hydroxide (VWR cat #VW 6730 or equivalent). To the solution was added Tween 20 (5.0 mL, Sigma cat #P-1379 or equivalent), and the mixture stirred gently.

Just prior to use 100 mL of this solution was diluted to 1.0 L with milliQ water.

Substrate solution: Substrate buffer was prepared by dissolving citric acid (4.20g, Malinckrodt cat #0627 or equivalent) and disodium hydrogen phosphate (7.10 g, Baker cat #3828-1 or equivalent) in 1.0 L of milliQ water. The pH was adjusted to 5.00 with 50% w/w sodium hydroxide (VWR cat #VW6730-3 or equivalent). Immediately prior to use an OPD substrate tablet (30 mg, Sigma cat #P-8412 or equivalent and 30% v/v hydrogen peroxide (40 μ L, Sigma cat #P-1379 or equivalent) were added to the substrate buffer 25.0 mL). The solution was wrapped in foil and mixed thoroughly.

4 NH_4SO_4 ; Sulfuric acid (53 mL, EM Science cat #SX1244-5 or equivalent) was slowly added to milliQ water (447 mL) and cooled to room temperature prior to use.

Equipment: Molecular Devices Model nu-max plate reader or equivalent. Scientific Products Model R4140 Oscillating table shaker and equivalent. Branson Model 5200 ultra-sonic bath or equivalent. Finnpiptette Model 4172317 multichannel pipeter or equivalent. Corning Model 25801 96 well disposable polystyrene Elisa Plates.

Prior to use and after each subsequent use the peptide pins were cleaned using the following procedure. Disruption buffer (2.0 L) was heated to 60° and placed in an ultra-sonic bath in a fume hood. To the disruption buffer was added dithiolthreitol (2.5 g, Sigma cat #D-0632 or equivalent). The peptide pins were sonicated in this medium for 30 min, washed thoroughly with milliQ waster, suspended in a boiling ethanol bath for 2 min, and air-dried.

Blocking buffer (200 μ L) was added to a 96 well disposable polystyrene Elisa plate and the peptide pins suspended in the wells. The peptide pins and plate were incubated for 2 h at room temperature on an oscillating table shaker. The plates and peptide pins were washed with PBS/Tween 20 (four times). To each well was added a 20 μ g/ml concentration of cA2 antibody (diluted with blocking buffer, 175 μ L/well). TNF competition was done by incubation of TNF α (40 μ g/ml) and cA2 (20 μ g/ml) in BSA/ovalbumin/BBS for three hours at room temperature. The peptide pins were suspended in the plate and incubated at 4° overnight. The peptide pins and plate were washed with PBS/Tween 20 (four

times). To each well was added anti-human goat antibody conjugated to horseradish peroxidase (diluted with blocking buffer to 1/2000, 175 μ L/well, Jackson ImmunoResearch Labs). The peptide pins were suspended in the plate, and incubated for 1 h at room temperature on a oscillating table shaker. The plates and peptide pins were washed with PBS/Tween 20 (four times). To each well added freshly prepared substrate solution (150 μ L/well), the peptide pins were suspended in the plate and incubated for 1 h at room temperature on an oscillating table shaker. The peptide pins were removed and to each well is added 4N H₂SO₄ (50 μ L). The plates were read in a Molecular Devices plate reader (490 nm, subtracting 650 nm as a blank), and the results are shown in Figures 14A and 14B, as described above.

EXAMPLE XIV

Production Mouse Anti-Human TNF mAb Using TNF Peptide Fragments

Female BALB/c mice, as in Example I above, are injected subcutaneously and intraperitoneally (i.p.) with forty μ g of purified E. coli-derived recombinant human TNF (rhTNF) fragments comprising anti-TNF epitopes of at least 5 amino acids located within the non-contiguous sequence 59-80, 87-108 or both residues 59-80 and 87-108 of TNF (of SEQ ID NO:1), as presented above, emulsified with an equal volume of complete Freund's adjuvant (obtained from Difco Laboratories) in 0.4 ml is into a mouse. One week later, a booster injection of 5 μ g of these rhTNF fragments in incomplete Freund's adjuvant is given i.p. followed by four consecutive i.p. injections of 10 μ g of TNF fragments including anti-TNF epitopes including amino acids from residues 59-80, 87-108 or both 59-80 and 87-108 of hTNF α (of SEQ ID NO:1) without adjuvant. Eight weeks after the last injection, the mouse is boosted i.p. with 10 μ g of TNF.

Four days later, the mouse is sacrificed, the spleen is obtained and a spleen cell suspension is prepared. Spleen cells are fused with cells of the nonsecreting hybridoma, Sp2/0 (ATCC CRL1581), at a 4:1 ratio of spleen cells to Sp2/0 cells, in the presence of 0.3 ml of 30% polyethylene glycol, PEG 1450. After incubation at 37°C for 6 hours, the fused cells are distributed in 0.2 ml aliquots into 96-well plates at concentrations of 2×10^4

SP2/0 cells per well. Feeder cells, in the form of 5×10^4 normal BALB/c spleen cells, are added to each well.

The growth medium used consisted of RPMI-1640 medium, 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin (GIBCO Laboratories) and, for selection, hypoxanthine-aminopterin-thymidine (HAT) (Boehringer Mannheim).

A solid-phase radioimmunoassay (RIA) is employed for screening supernatants for the presence of mAbs specific for rhTNF α fragments including portions of residues 59-80, 87-108 or both 59-80 and 87-108 of hTNF α (of SEQ ID NO:1). This assay is described in Example II, above. The background binding in this assay is about 500 cpm. A supernatant is considered positive if it yielded binding of 2000 cpm or higher.

Of the supernatants screened, one or more positive supernatants are routinely identified by RIA. Of these positive supernatants, the highest binding (as shown by the higher cpm values) are subcloned at limiting dilution on mouse feeder cells. Upon further analysis of the supernatants in neutralization assays, routinely one or more antibodies are found to have potent inhibiting and/or neutralizing activity. These positive and inhibiting and/or neutralizing hybridoma lines are then selected and maintained in RPMI-1640 medium with 10% FBS (GIBCO), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin.

EXAMPLE XV

Production of Murine and chimeric antibodies, fragments and regions from TNF Peptides

Murine and chimeric antibodies, fragments and regions are obtained by construction of chimeric expression vectors encoding the mouse variable region of antibodies obtained in Example XIV and human constant regions, as presented in Examples IV-IX above.

The resulting chimeric A2 antibody is purified from tissue culture supernatant by Protein A-Sepharose chromatography. The supernatant is adjusted to 0.1M Tris, 0.002M EDTA, pH 8.0 and loaded on a Protein A-Sepharose column equilibrated in the same

buffer. The IgG is then eluted with 0.1M citrate, pH 3.5, neutralized with 1M Tris, and dialyzed into phosphate buffered saline (PBS).

The purified murine and chimeric antibodies, fragments and regions are evaluated for its binding and inhibiting and/or neutralizing activity.

EXAMPLE XVI

In Vitro Activity and Neutralization Efficacy of a Chimeric Anti-TNF Antibody

Both the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, are determined to have potent TNF-inhibiting and/or neutralizing activity, as shown for example, in the TNF cytotoxicity assay described above, expressed as the 50% Inhibitory Dose (ID50).

In this same experimental system, three other murine anti-TNF α antibodies (termed TNF-1, TNF-2 and TNF-3) of comparable binding affinity to TNF are found to have ID50 values of 1-2 orders of magnitude greater, and thus have significantly less potent in neutralization, than both the murine and chimeric anti-TNF α antibodies of the present invention.

The ability of both the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, to inhibit or neutralize human TNF α bioactivity in vitro is tested using the bioassay system described above. Cultured cells producing the murine or chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, are incubated with 40 pg/ml natural (Genzyme, Boston, MA) or recombinant (Suntory, Osaka, Japan) human TNF with or without antibody overnight as above, and cell death is measured by vital staining. As expected, both the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, inhibited or neutralized both natural and rhTNF in a dose-dependent manner in the cytotoxicity assay. Such inhibiting and/or neutralizing potency, at antibody levels below 1 μ g/ml, can easily be attained in the blood of a subject to whom the antibody is administered. Accordingly, such highly potent inhibiting and/or neutralizing anti-TNF antibodies,

in particular the chimeric antibody, are preferred for therapeutic use in TNF α -mediated pathologies or conditions.

5 The ability of cA2 to inhibit or neutralize TNF-induced IL-6 secretion is evaluated using cultured human diploid FS-4 fibroblasts. The results are expected to show that both murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, are effective in blocking IL-6 secretion in cells that had been incubated overnight with TNF. TNF-induced IL-6 secretion is not inhibited in the absence of a mAb or in the presence of a control mAb specific for an irrelevant antigen.

10 The ability of TNF to activate procoagulant and adhesion molecule activities of endothelial cells (EC) is thought to be an important component of pathology pathophysiology. In particular, this may be associated with the vascular damage, disseminated intravascular coagulation, and severe hypotension that is associated with the sepsis syndrome. Therefore, the ability of both the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, to block TNF-induced activation of cultured human umbilical vein endothelial cells (HUVEC) is evaluated. TNF stimulation of procoagulant activity is determined by exposing intact cultured HUVEC cells to TNF (with or without antibody) for 4 hours and analyzing a cell lysate in a human plasma clotting assay. The results are expected to show the expected upregulation by TNF of HUVEC procoagulant activity (reflected by a decreased clotting time). Both the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, are expected to effectively inhibit or neutralize this TNF activity in a dose-dependent manner.

20 25 30 In addition to stimulating procoagulant activity, TNF also induces surface expression of endothelial cell adhesion molecules such as ELAM-1 and ICAM-1. The ability of both the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, are expected to inhibit or neutralize this activity of TNF is measured using an ELAM-1 specific detection radioimmunoassay. Cultured HUVEC are stimulated with 250 ng/ml rhTNF (Dainippon, Osaka, Japan) with or without antibody at 37°C overnight in a 96-well plate format.

Surface expression of ELAM-1 is determined by sequential addition of a mouse anti-human ELAM-1 mAb and ¹²⁵I-labelled rabbit anti-mouse immunoglobulin second antibody directly to culture plates at 4°C.

5 TNF is expected to induce the expression of ELAM-1 on the surface of cultured HUVEC cells, and this activity is again expected to be effectively blocked in a dose-related manner by both the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV.

10 Finally, TNF is known to stimulate mitogenic activity in cultured fibroblasts. Both the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, are expected to inhibit or neutralize TNF-induced mitogenesis of human diploid FS-4 fibroblasts cultures, confirming the potent inhibiting and/or neutralizing capability of
15 both the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV against a broad spectrum of *in vitro* TNF biological activities.

EXAMPLE XVII

In Vivo Activity and Efficacy of cA2 Antibody

20 An *in vivo* model wherein lethal doses of human TNF are administered to galactosamine-sensitized mice (Lehmann, V. et al., *supra*) is adapted for testing the capability of both the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, to inhibit or neutralize TNF *in vivo*. An i.p. challenge with 5 μ g (0.25 mg/kg) of rhTNF resulted
25 in 80-90 percent mortality in untreated control animals and in animals treated i.v. 15-30 minutes later with either saline or 2 mg/kg control antibody (a chimeric IgG1 derived from murine 7E3 anti-platelet mAb). In contrast, treatment with both the murine
30 and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, is expected to reduce mortality to 0-30 percent with 0.4 mg/kg of antibody, and to 0-10 percent with 20 mg/kgs. These expected results indicate that both
35 the murine and chimeric anti-TNF α antibodies of the present invention, as obtained according to Examples XIV and XV, are capable of inhibiting and/or neutralizing the biological activity of TNF *in vivo* as well as *in vitro*.

EXAMPLE XVIII

Clinical Activity and Efficacy of cA2 Antibody

Chimeric IgG1 anti-human TNF monoclonal antibody cA2 was administered to healthy male human volunteers as patients. One hour after receiving 4 ng/kg of an NIH reference endotoxin, the volunteers were administered either saline, as a control, or 0.01, 0.10 or 10 mg/kg of cA2 in a pharmaceutically acceptable form. TNF levels in serum were measured over time and were found to show a dose dependent decrease in peak TNF levels with no TNF being detected in volunteers receiving a 10 mg/kg dose of cA2. Accordingly, therapy with an anti-TNF antibody of the present invention is expected to inhibit TNF-mediated effects in humans.

Patients receiving endotoxin developed pronounced leukopenia thought to be due to margination of white blood cells. As the white blood cells become activated, they can attach to endothelial receptors with resultant endothelial damage. At doses of 1.0 to 10.0 mg/kg, this leukopenia is prevented, whereas, at 0.01 and 0.1 mg/kg dosages, a drop in white cell count was observed. The drop was most pronounced among the polymorph cell line. In all patients there was a subsequent leukocytosis, which was unchanged by treatment with anti-TNF anti-body cA2. This blocking effect on white blood cell margination is expected to represent a protective effect against the endothelial damage associated with TNF. It is expected in the art that this TNF-related endothelial damage plays a significant role in the morbidity and mortality associated with sepsis, and it is therefore expected that the anti-TNF antibodies of the present invention will provide a protective effect against these damaging effects, as presented herein.

EXAMPLE XIX:

CLINICAL TREATMENT OF RHEUMATOID ARTHRITIS BY A ANTI-TNF ANTIBODY OR FRAGMENT OF THE PRESENT INVENTION

A Phase I open label study of the present invention as a chimeric anti-TNF monoclonal antibody for the treatment of patients with severe refractory rheumatoid arthritis was conducted.

Nine patients were enrolled in the study. The first five patients were treated with chimeric anti-TNF antibody (cA2), 10 mg/kg as a single dose infused over a period of two hours. These

patients were subsequently retreated with a second infusion of 10 mg/kg on day 14 of the study. The second group of five patients received an infusion of 5 mg/kg on the first day of the study. They were then treated with additional infusions of 5 mg/kg on days 5, 9, and 13. Four of the planned five patients in this second group have been treated to date.

Preparation, Administration, and Storage of Test Material

The chimeric monoclonal anti-TNF antibody was supplied in single-use glass vials containing 20 mL with 100 mg of anti-TNF (5 mg/mL). The anti-TNF antibody was stored at 2-8°C. Prior to infusion, the antibody was withdrawn from the vials and filtered through a low-protein-binding 0.22 μ m filter. This filtered antibody was then diluted to a final volume of 300 mL with normal saline. The 300 mL antibody preparation was then infused via an in-line filter over a period of not less than two hours.

Prior to each repeat infusion of study medication a test dose of 0.1 mL of the infusion was diluted in 10 mL of normal saline and administered by slow IV push over 5 minutes. The patient was observed for 15 minutes for signs or symptoms of an immediate hypersensitivity reaction. If no reaction was observed in this time period, the full dose was administered as described above.

Administration Protocol

Group 1 (patients 1-5): a total of 2 infusions, on day 1 and day 15 of the trial; dosage 10 mg/kg on each occasion;

Group 2 (patients 6-9): a total of 4 infusions, on days 1, 5, 9 and 13 of the trial; dosage 5 mg/kg on each occasion.

All infusions were administered iv over 2 hours in a total volume of cA2 + saline of 300 ml.

Infusions subsequent to the first in any patient were preceded by a small test dose administered as an iv push.

All patients had at least three years of disease activity with rheumatoid arthritis. The patients ranged in age from 23 to 63. All patients had failed therapy with at least three different DMARD (Disease Modifying Anti-Rheumatic Drug). Six of the nine patients had serum rheumatoid factors, and all nine patients had erosions present on X-rays.

Clinical Monitoring

Patients were monitored during and for 24 hours after infusions for haemodynamic change, fever or other adverse events.

Clinical and laboratory monitoring for possible adverse events was undertaken on each follow-up assessment day.

Clinical response parameters were performed at the time-points as specified in the flow chart present in Table 8. These evaluations were performed prior to receiving any infusions.

Clinical response studies will be comprised of the following parameters:

1. Number of tender joints and assessment of pain/tenderness
The following scoring will be used:

0 = No pain/tenderness

1 = Mild pain. The patient says it is tender upon questioning.

2 = Moderate pain. The patient says it is tender and winces.

3 = Severe pain. The patient says it is tender and winces and withdraws.

2. Number of swollen joints

Both tenderness and swelling will be evaluated for each joint separately. MCP's, PIP's etc. will not be considered as one joint for the evaluation.

3. Duration of morning stiffness (in minutes)

4. Grip strength

5. Visual analog pain scale (0-10 cm)

6. Patients and blinded evaluators will be asked to assess the clinical response to the drug. Clinical response will be assessed using a subjective scoring system as follows:

5 = Excellent response (best possible anticipated response)

4 = Good response (less than best possible anticipated response)

3 = Fair response (definite improvement but could be better)

2 = No response (no effect)

1 = Worsening (disease worse)

Measurement of index of disease activity is scored according to the following table 9.

Table 4. Clinical characteristics of patients 1-5

Patient Number	Age/Sex	Disease Duration (years)	Rheumat. Factor	Erosions/ Nodules	Previous Treatment (DMARDs only)	Concomitant Anti-rheumatic Therapy
01	48/F	7	+ve	+ve/+ve	*Sal,DP,Myo,Aur, MTX,Aza,Chl.	**Pred 5 mg
02	63/F	7	-ve	+ve/-ve	Sal,Myo,DP.	Para 1-2g
03	59/M	3	+ve	+ve/-ve	Aur,Chl,Myo, MTX,Sal.	Pred 10 mg, Ind 225 mg
04	56/M	10	+ve	+ve/-ve	Myo,DP,Aza,Sal.	Pred 12.5 mg, Ibu 2g, Para 1-2g
05	28/F	3	+ve	+ve/-ve	Myo,Sal,DP,Aza.	Pred 8mg, Para 1-2g, Cod 16 mg

*Sal = Sulphasalazine; DP = D-penicillamine; Myo = Myocrisin; Aur = auranofin; MTX = methotrexate; Aza = azathioprine; Chl = hydroxychloroquine. **Pred = prednisolone (dosage/day); Para = paracetamol; Ind = indomethacin; Ibu = ibuprofen; Cod = codeine phosphate.

Table 5. Clinical characteristics of patients 6-9

Patient Number	Age/Sex	Disease Duration (years)	Rheumat. Factor	Erosions/ Nodules	Previous Treatment (DMARDs only)	Concomitant Anti-rheumatic Therapy
06	40/M	3	+ve	+ve/-ve	*Sal,Chl,Aur.	**Nap 1 g
07	54/F	7	-ve	+ve/-ve	DP,Myo,Sal,Aza, MTX.	Para 1-2g, Cod 16-32 mg.
08	23/F	11	+ve	+ve/-ve	Chl,Myo,Sal, MTX, Aza.	Pred 7.5 mg, Dicl 100 mg, Para 1-2g, Dext 100-200 mg.
09	51/F	15	-ve	+ve/+ve	Myo,Chl,DP, MTX.	Pred 7.5 mg, Dicl 125 mg, Para 1-3g.

*Sal = Sulphasalazine; Chl = chloroquine or hydroxychloroquine; Aur = auranofin; DP = D-penicillamine; Myo = Myocrisin; Aza = azathioprine; MTX = methotrexate. **Nap = naprosyn (dosage/day); Para = paracetamol; Cod = codeine phosphate; Pred = prednisolone; Dicl = diclofenac; Dext = dextropropoxyphene.

Table 6. Disease activity at entry for patients 1-5

Patient Number	Morning Stiffness (mins)	Pain (0-10 cm on VAS)	Number Swollen Joints (0-28)	Ritchie Articular Index (0-69)	Grip Strength L/R (mm/Hg; max 300)	ESR (mm/hr normal ranges: F < 15; M < 10)	CRP (mg/l; normal range: < 10)	IDA (range: 1-4)
01	60	3.9	19	30	108/107	35	5	2.67
02	20	2.7	25	31	67/66	18	14	2.0
03	90	4.9	14	16	230/238	48	44	2.5
04	30	6.9	17	12	204/223	24	35	2.33
05	90	5.7	28	41	52/89	87	107	3.0

Table 7. Disease activity at entry for patients 6-9

Patient Number	Morning Stiffness (mins)	Pain (0-10 cm on VAS)	Number Swollen Joints (0-28)	Ritchie Articular Index (0-69)	Grip Strength L/R (mm/Hg; max 300)	ESR (mm/hr normal ranges: F < 15; M < 10)	CRP (mg/l; normal range: < 10)	IDA (range: 1-4)
5								
06	120	5.0	3	4	260/280	23	33	2.33
10								
07	105	7.4	27	31	59/80	25	10	2.83
08	270	9.3	17	37	73/125	35	31	3.17
09	180	4.5	20	26	53/75	15	33	2.5

5
 10
 15

All patients have tolerated the infusions of chimeric anti-TNF α and no serious adverse reactions have been observed. Specifically, no episodes of hemodynamic instability, fevers, or allergic reactions were observed in association with the infusions. Patients have not experienced any infections.

20

Although this is a non-blinded study, all patients have experienced improvement in their clinical assessments of disease status, as well in biochemical parameters of inflammation measured in their serum.

25
 30

Clinical assessments, including the duration of early morning stiffness; the assessment of pain on a visual analogue scale; total count of swollen joints; Ritchie articular index (a scaled score which assesses the total number of tender joints and the degree of joint tenderness); and Index of Disease Activity (a scaled score which incorporates several clinical and laboratory parameters), showed impressive improvements compared to baseline. These improvements were typically in the range of an 80% drop from the baseline score; a degree of improvement which is well beyond the amount of improvement that can be attributed to placebo response. In addition, the duration of these improvements was for six to eight weeks in most cases, a duration of response far longer than would be anticipated from a placebo.

35
 40

The improvements in clinical assessments were corroborated by improvements in biochemical inflammatory parameters measured in serum. The patients showed rapid drops of serum C-reactive protein, usually in the range of 80% from the baseline. Reductions in the erythrocyte sedimentation rate, usually in the range of 40%, were also observed. Circulating soluble TNF receptors were also decreased following therapy. The durations of the biochemical responses were similar to the duration of the clinical responses.

Preliminary assessment of immune responses to the chimeric anti-TNF antibody has shown no antibody response in the first four patients.

In summary, the preliminary evaluation of the results of this Phase I trial indicate that treatment of patients with advanced rheumatoid arthritis with anti-TNF monoclonal antibody of the present invention is well tolerated and anti-TNF treatment is associated with rapid and marked improvement in clinical parameters of disease activity, including early morning stiffness, pain, and a number of tender and swollen joints; and is accompanied by improvement of biochemical parameters of inflammation.

Although this was an open label study, the magnitude of the clinical improvements is well beyond the degree of improvement that would be anticipated from a placebo response, such that the present invention is shown to have significant clinical efficacy for treating rheumatoid arthritis.

Table 8A

Flowchart for CHIMERIC ANTI-TNF STUDY C0168TRA

Group I (10 mg/kg at day 1 and day 14)

	Pr e Sc r	Scr een ing	Wk d1	0 d 2	Wk 1	W k 2 d 1 4	W k 3	W k 4	W k 6	W k 8	
Consent	x										
Demography		x									
Physical Examination		x								x	
Pregnancy Test		x									
Weight		x	x			x				x	
Vital Signs		x	x*	x	x	x*	x	x	x	x	

5

Anti-TNF Infusion			x			x					
Labs, see Chart		x	x'	x	x	x'	x	x	x	x	
Clinical (Safety)				x	x	x'	x	x	x	x	
Clinical (Response)		x	x'		x	x'	x	x	x	x	
Synovial biopsy		x			x7	-					
Response evaluation										x	

	Scr een ing	Wk d1	0 d2	Wk 1	Wk 2 d1 4	Wk 3	Wk 4	Wk 6	Wk 8	
Hematology + ESR	x	x'		x	x'	x	x	x	x	
Biochemistry	x	x'		x	x'	x	x	x	x	
Urinalysis		x'		x	x'	x	x	x	x	
CRP + RF		x'		x	x'	x	x	x	x	
Serum Cytokines		x'		x	x'	x	x	x	x	
PBL		x	x	x						
Pharmacokinetics		x [#]	x [#]		x ^{\$}					
HACA response		x'		x	x'	x	x	x	x	x

x* = Vital signs will be obtained prior to infusion, every 30 minutes during the infusion and every 30 minutes for 2 hours after the infusion.

x' = Needs to be done prior to the infusion.

x# = Serum samples will be obtained prior to the infusion and at 1, 2, 4, 8, 12, and 24 hours after the end of the infusion.

x\$ = Serum samples will be obtained to the infusion and at 2 hours after the end of the infusion.

Table 8B

Flowchart for CHIMERIC ANTI-TNF STUDY C0168TRA

Group II2 (mg/kg every 4 days, 4 times total)

	Pr e Sc r	Scr een ing	d1	wk d2	0 d5	+ d9	1 d13	wk 2	wk 3	wk 4	wk 6	wk 8	
5	Con- sent	x											
	Demo - gra- phy	x											
10	Phys - ical exam	x										x	
15	Preg - ancy test	x											
	Wei- ght	x	x		x	x	x					x	
20	Vita l sign s	x	x*	x	x*	x*	x*	x	x	x	x	x	
25	Anti - TNF Infu - sion		x		x	x	x						

5

Labs
,
see
char
t

x

x'

x

x'

x'

x'

x

x

x

x

x

x

10

Clin
-
ical
Saf-
ety

x

x'

x'

x'

x

x

x

x

x

15

Clin
-
ical
Res-
pons
e

x

x'

x'

x

x

x

x

x

20

Syno
-
vial
Bio-
psy

x

x7

25

Res-
pons
e
Eval
-
uat-
ion

x

	sc r ee n in g	d1	wk d2	0 d5	+	1 d1 3	wk 2	wk 3	wk 4	wk 6	wk 8
Hematology + ESR	x	x'			x'		x	x	x	x	x
Biochemist ry	x	x'			x'		x	x	x	x	x
Urinalysis		x'			x'		x	x	x	x	x
CRP + RF		x'			x'		x	x	x	x	x
Cytokines		x'			x'		x	x	x	x	x
PBL		x	x		x				x		
Pharmacoki netics		x#	x#	x\$	x\$	x\$					
HACA Response		x'			x'		x	x	x	x	x

x* = Vital signs will be obtained prior to infusion, every 30 minutes during the infusion and every 30 minutes for 2 hours after infusion.

x' = Needs to be done prior to the infusion.

x# = Serum samples will be obtained to the infusion and at 1, 2, 4, 8, 12, and 24 hours after the end of the infusion.

x\$ = Serum samples will be obtained prior to the infusion and at 2 hours after the end of the infusion.

Table 9
Measurement of the index of disease activity (DA)
Variables of Disease Activity

5	IDA score	Morning Stiffness (min)	Pain (VAS, cm) *	Grip Strength (mmHg)	Ritchie Articular Index	Hemoglobin (g/dl)		ESR
						Male	Female	
	1	<10	0-2.4	>200	0	≥14.1	≥11.7	0-20
	2	10-30	2.5-4.4	50-200	1-7	13-14	10.8-11.6	21-45
10	3	31-120	4.5-6.4	30-49	8-17	10-12.9	8.4-10.7	46-80
	4	>120	6.5-10	<30	>18	≤9.9	≤8.3	≥81

*Pain was measured on a visual analog scale (VAS) 0-10 cm.

Conclusions (1)

15 Safety of anti-TNF in RA

Anti-TNF was safe and very well tolerated:

- no haemodynamic, febrile or allergic episodes;
- no infections;
- no clinical adverse events;
- 20 - a single laboratory adverse event only, probably
unrelated to anti-TNF.

Conclusions (2)

Efficacy of anti-TNF in RA

Anti-TNF therapy resulted in:

- 25 - rapid and marked improvements in EMS, pain and articular
index in most patients;
- slower but marked improvement in swollen joint score,
maximal by 3-4 weeks;
- rapid and impressive falls in serum CRP, and a slower
30 fall in ESR;
- normalization of CRP and ESR in some patients;
- rapid falls in serum C4d (a complement breakdown product)
and IL-6 in patients where these indices were elevated at entry.

35 Duration of clinical improvements variable, with rebound in
some patients at 6-8 weeks.

Accordingly, the present invention has been shown to have
clinical efficacy in human patients for treating TNF involved
pathologies using TNF monoclonal antibodies of the present
invention, such as for treating rheumatoid arthritis.
40 Additionally, the human clinical use of TNF antibodies of the

present invention in humans is also shown to correlate with in vitro data and in vivo animal data for the use of anti-TNF antibodies of the present invention for treating TNF-related pathologies.

EXAMPLE XX:

Treatment of Crohn's Disease in Human Using Anti-TNF α Antibodies.
Case history SB.

This 16 year old patient has a history of Crohn's disease since age 12. She was suffering from diarrhoea, rectal blood loss, abdominal pain, fever and weight loss. She showed perianal lesions, severe colitis and irregularity of the terminal ileum. She was treated with prednisolone (systemic and local) and pentasa. This resulted in remission of the disease, but she experienced extensive side effects of the treatment. She experienced severe exacerbations at age 12 and 12 yrs, 5 months, (ImmunanTM added), 12 yrs, 9 months, 13 yrs, 5 months, and 14 yrs, 10 months. She experienced severe side effects (growth retardation, morbus Cushing, anemia, muscle weakness, delayed puberty, not able to visit school).

At 15 yrs, 11 months, she was diagnosed with a mass in the right lower quadrant. She had a stool frequency of 28 time per week (with as much as 10 times per day unproductive attempts). The Crohn's index was 311, the pediatric score 77.5. The sedimentation rate was elevated. Albumen and hemoglobin reduced. Before the first treatment the score was 291 and pediatric score was 60, and she would possibly have to lose her colon. She was infused on compassionate grounds with 10 mg/kg cA2, without any side effects noticed. One week after treatment her sedimentation rate was reduced from 66 to 32 mm. The Crohn's index was 163 and pediatric score 30. She was reported to feel much better and the frequency of the stools was reduced greatly. ^{There} ~~Thee~~ was apparently no more diarrhoea, but normal faeces. On October 15th, before the second infusion she had gained weight, had a sedimentation rate of 20 mm, an albumen of 46 h/l, Crohn's index 105, pediatric score 15. There seemed to be improvement on video endoscopy. A second infusion was performed at 16 yrs.

The patient was greatly improved after the second infusion. A endoscopy showed only 3 active ulcers and scar tissue.

This is in contrast with her colon on admission when the thought was that her colon should be removed. This case history shows a dramatic improvement of severe Crohn's disease upon treatment with cA2 anti-TNF antibody.

5 Table 11:

CASE HISTORY SB

	11y, 8m:	physical	Diarrhoea, rectal blood loss, abdominal pain,
10		examination:	fever (40%) weight loss perianal lesions
		sigmoidoscopy:	severe colitis, probably M. Crohn
		enterolysis:	irregularity terminal ileum
15		Therapy:	prednisolone 10 mg 3 dd. Pentasa 250 mg 3 dd. enema (40 mg prednisone, 2g 5 ASA) ml 1 dd.
		Result:	remission, however: extensive side effects of prednisone and stunting growth
		Action:	prednisone
20	11y, 11m	exacerbation	same clinical picture as 11 y, 8 m
		sigmoidoscopy:	recurrence of colitis (grade IV) in last 60 cm <u>and</u> anus.
25		Therapy:	prednisolone 40 mg 1 dd Pentasa 500 mg 3 dd enema 1 dd
		Result:	little bit better
30	12y, 5m:	severe exacerbation; despite intensive treatment	
		sigmoidoscopy:	extensive perianal and sigmoidal lesions; active disease
		Therapy:	continued + Immuran(TM) 25 mg 1 dd
		Result:	slight improvement however still growth retardation, cushing, anaemia, muscle weakness.
35		Action:	prednisone
	12y, 9m:	exacerbation	
		sigmoidoscopy:	extensive (active) colitis, polyps
40		Action:	prednisone: 30 mg 1 dd, Immuran(TM) 50 mg 1 dd, Pentasa 500 mg 3 dd, enema 2 dd
		Result:	still needs enema's with prednisone <u>and</u> oral prednisone. delayed puberty, stunting growth
45	14y, 10m:		severe exacerbation, weight loss, abdominal pain, fever.
		ileoscopy:	active colitis (grade IV), perianal lesions. Terminal ileum normal.
		Result:	No remission still fever, poor appetite, weight loss, diarrhea, not able to visit school

Important Findings:

14y, 11m:

151.9 cm; 34 kg
T=38°C, Abdominal mass in right lower quadrant
stool frequency 28 per week
(however goes 10-15 times a day but most often without success)
ESR 55 mm; Hb 6.2 mmol/l
Ht 0, 29 1/1; alb. 38.4 g/l
Crohn's Dis. Act. Index: 311
Pediatric score: 77.5

14y, 11.2m:
(before 1st infusion)

151,8 cm: 34.6 kg
Crohn's Dis Act Index: 291
Pediatric score: 60

14y, 11.4m:

151,8 cm: 34.6 kg
ESR 32 mm; Hb 5.7 mmol/l
Crohn's Dis Act Index: 163
pediatric score: 30

15y, 0m
(before 2nd infusion)

152,1 cm: 34.8 kg
feels like she has never felt before.
Parents also very enthusiastic
ESR 30 mm; Hb 6, 3 mol/l
Ht 0,32 11; Alb 46 g/l
Crohn Dis Act Index: 105
Pediatric Score: 15
Videoendoscopy: Their seems to be improvement

No problems or side effects observed during and following infusion.

Accordingly, Anti-TNF antibodies according to the present invention, as exemplified by cA2 are shown to provide successful treatment of TNF related pathologies, as exemplified by Crohn's disease, in human patients with no or little side effects.

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily
5 modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the
10 teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance
15 presented herein, in combination with the knowledge of one of ordinary skill in the art.